Understanding the Biology of HPV E6 Oncoprotein in Cervical Cancer

Thesis

Understanding the Biology of HPV E6 Oncoprotein in Cervical Cancer

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Abstract

The high-risk Human Papillomavirus (HPV) E6 oncoprotein is known to contribute to human malignancy by targeting several of its cellular substrates for degradation through the ubiquitin-proteasome pathway. Whilst E6AP plays a critical role in targeting p53, its role in the degradation of many other E6 substrates is unclear because loss of E6AP also induces E6 degradation. To examine this further, we used CRISPR-edited E6AP knockout (E6AP K/O) cells to perform E6 degradation assays in the presence of a catalytically inactive mutant form of E6AP, thus ensuring the stabilization of E6, but with the ligase itself being functionally inactive. We found that E6 can mediate the degradation of several PDZ domain-containing proteins independently of E6AP ubiquitin ligase activity. Using this system, we also aimed to identify the ligases potentially responsible for degrading E6 in the absence of E6AP by performing a high-throughput human siRNA library screen against ubiquitin ligases, using expression of GFP-tagged HPV-18E6 as the reporter. We found a number of ubiquitin ligases whose knockdown rescued E6 protein levels, with FBXO4 being the best candidate. We also found that loss of E6AP also induces a dramatic increase in the levels of phosphorylated E6 (pE6), despite the expected overall reduction in total E6 protein levels. Phosphorylation of E6 requires transcriptionally active p53 and occurs in a manner that is dependent upon DNA PK. These results identify a novel feedback loop, where loss of E6AP results in upregulation of p53, leading to increased levels of E6 phosphorylation, which, in turn. correlates with increased association with 14-3-3 and inhibition of p53 transcriptional activity.

Finally, we show that knockdown of E6AP in HPV-positive cervical cancer-derived cells causes a marked decrease in E7 protein levels. This is due to a decrease in the E7 half-life and occurs in a proteasome-dependent manner. In an attempt to define the underlying mechanism, we show that E7 can also associate with E6AP, albeit in a manner different from that of E6. In addition, we show that E6AP-dependent stabilisation of E7 also leads to an increase in the degradation of E7’s cellular target substrates. Interestingly, ectopic over-expression of E6 protein results in lower levels of E7 protein, through E6's sequestration of E6AP, demonstrating a surprising interplay between E6 and E7, in manner that is mediated by the E6AP ubiquitin ligase.

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List of Abbreviations

HPV – human papilloma virus
IARC - International Agency for Research on Cancer
ORFs - open reading frames
URR – upstream regulatory region
HSPGs – heparin sulphate proteoglycans
ECM – extracellular matrix
ESCRT – endosomal sorting complexes required for transport
TSG101 – tumour susceptibility gene 101
VPS4 – vacuolar protein sorting-associated protein 4A
ALIX – ALG-2-interacting protein X
TGN – trans-Golgi network
PML – promyelocytic leukemia nuclear (bodies)
ori – origin of replication
E6AP – ubiquitin ligase E6 associated protein
LSIL – low-grade squamous intraepithelial lesions
CIN1/2/3 – cervical intra-epithelial neoplasia grade 1/2/3
HSIL – high-grade squamous intraepithelial lesions
CFS – common fragile sites
DSBs – double-stranded DNA breaks
DNA-PK – DNA-dependent protein kinase
ROS – reactive oxygen species
RONS – reactive oxygen nitrogen species
CD – conserved domain
CKII – casein kinase II
SCF – skp-Cullin-F box
PTPN14 – protein tyrosine phosphatase non-receptor type 14
P/CAF – p300/CBP-associated factor
BAZ2B- Bromodomain adjacent to Zinc finger domain protein 2B
RNF5- Ring Finger protein 5
FBOX4- F-Box protein 4
FBXL19- F-Box and leucine rich repeat protein 19
ASB1- Ankyrin repeat and SOCS box protein 1
NF-kβ – nuclear factor kappa-light-chain-enhancer of activated B cells
CDKs – cyclin-dependent kinases
TGF-β – tumour growth factor β
PBM- PDZ-binding motif
PKA-Protein Kinase A
ATP- Adenosine tri phosphate
HSV- Herpes simplex virus
KSHV- Kaposi sarcoma associated virus
UPS- Ubiquitin proteasome system
RING- Really interesting new gene
HECT- homologous to E6-AP C-terminus
SCF- SKp1-Cul1-F-box
APC/C- Anaphase promoting complex/cyclosome
Ub- Ubiquitin
EBV- Epstein-Barr Virus
HERC2- HECT and RLD domain containing E3 ubiquitin ligase 2
MAPK6- Mitogen-activated protein kinase-6
NEUL4- Neuralized- like protein 4
TRIM- Tripartite motif containing
GPCA- *Gaussia princeps* luciferase complementation assay
NFX1- Nuclear transcription factor, X-box binding-1
ZO-1/2- Zona Occludens 1/ 2
ATM- Ataxia telangiectasia mutated
ATR- Ataxia telangiectasia and RAD3-related
FADD- Fas-associated death domain
TNF R1- Tumour necrosis factor receptor 1
DDR- DNA Damage Response
FA- Fanconi Anæmia

ZNRF3- Zinc and ring finger 3
PI- Propidium Iodide
ELISA- Enzyme-linked immunoassay
FACS- Fluorescence activated cell sorting
CRISPR- Clustered regularly interspaced short palindromic repeats

Introduction

HPV and Cervical cancer

Cervical cancer is the second most common type of cancer affecting women worldwide and accounts for the vast majority of HPV-attributable cancer cases worldwide. Globally, approximately 530,000 new cases are detected every year, with 266,000 deaths worldwide. Notably, half of the patients are women below 50 years of age and most are from developing countries. The majority of cervical cancer cases occur in South-Eastern Asia - India carries the most significant burden; followed by sub-Saharan Africa and Latin America. However, about 80-90% of HPV infections are transient, usually resolving spontaneously in 1-2 years, and are asymptomatic (Serrano, Brotons et al. 2018).

More than 200 different genotypes of HPV have been identified, classified into five genera: α, β, γ, ν and μ, based on differences in their life cycles, their DNA sequences and also in their disease association (Doorbar 2006, Bernard, Burk et al. 2010, Doorbar, Egawa et al. 2015). It is now believed that viruses from the beta and gamma genera complete their life-cycle, causing asymptomatic infections in immunocompetent individuals, without causing any apparent disease phenotype (Forslund 2007, Nindl, Gottschling et al. 2007, Gottschling, Goker et al. 2009, Bottalico, Chen et al. 2011). HPVs from the alpha genus are categorized as either cutaneous types or mucosal types (Bernard, Burk et al. 2010), based on their ability to infect basal epithelial cells of the skin or inner lining of tissues (Harwood, Spink et al. 1999, Burd 2003). The alpha type HPVs can also be grouped into high-risk and low-risk HPV types, based on their association with cervical cancer and precursor lesions. Low-risk HPV types include types 6, 11, 42, 43, and 44. The International Agency for Research on Cancer (IARC) has classified 12 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) as potential carcinogens, and these are known as high-risk types. Among all the high-risk HPV types, HPV-16 and HPV-18 stand out as markedly the most carcinogenic, and these are especially responsible for causing invasive cancer in humans (Harwood, Spink et al. 1999, zur Hausen 2002, Burd 2003, Doorbar, Egawa et al. 2015).

The high-risk virus types are estimated to cause about 5% of the cancer burden worldwide, which includes >99% of cervical cancers, 25%–60% of head-and-neck cancers, 70% of vaginal squamous cell carcinomas, 88% of anal cancers, 43% of vulvar and 50% of penile cancers (Gillison, Castellsague et al. 2014, Giuliano, Nyitray et al.

2015). Therefore, HPV continues to be an important subject for research, since the rate of HPV-related diseases is increasing day by day.

HPV Genome Structure

Despite their varying disease associations, all HPV types share some common features, including double-stranded genomic DNA with an average size of approximately 8000 base pairs, enclosed within an icosahedral capsid. The viral genome comprises eight to nine ORFs (Open Reading Frames), and is divided into the Early coding region (coding for core viral proteins such as E1, E2, E4, E5, E6 and E7) and the Late coding region (coding for the capsid proteins L1 and L2) as shown in Figure 1 (Zheng and Baker 2006).

Briefly, the E1 and E2 proteins of HPV act as factors that recognize the origin of replication and initiate viral genome amplification; the E2 protein also plays a role in regulating the transcription of other viral genes. E4, despite being known as an early gene, is involved in the late stages of the virus life cycle, and E5 is known to function during both the early and late phases of the viral life cycle. The E6 and E7 proteins target a number of negative regulators of the cell cycle, primarily p53 and pRb, respectively. E6 and E7 facilitate the stable maintenance of viral episomes during the viral life cycle and stimulate differentiating cells to re-enter S phase. Spliced variants E8^E2 is known to be involved in initiating vegetative viral genome amplification (Graham and Faizo 2017); E1^E4 expresses in the upper layers of stratified epithelia, coordinating with the onset of genome amplification but preceding the expression of L1 (Palefsky, Winkler et al. 1991, Doorbar, Foo et al. 1997) . The L1 and L2 proteins self-assemble into capsomers, which form icosahedral capsids around the viral genome during the generation of progeny virions (Fehrmann and Laimins 2003).

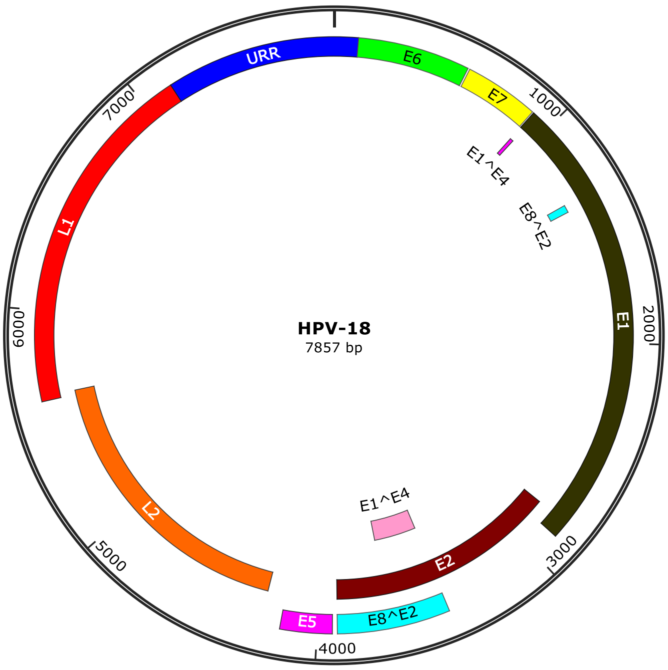


Figure 1. Representation of the HPV 16 Genome: The diagram shows the ORFs of the early (E) genes E1, E2, E4, E5, E6, E7 and splice variants E8^E2, E1^E4, which encode proteins necessary for viral replication, and late L1 and L2 genes, encoding proteins involved in virion packaging and release. The Upstream Regulatory Region (URR) contains the two major promoters that drive viral gene expression (P97 and P670) and control the viral replication and transcription.

HPV Viral life cycle

HPV viral life cycle as shown in Figure 2, is intimately linked with the differentiation program of the epithelium, and has been conventionally divided into two phases:

- **Early phase**: the period from virus entry into the host cells to the replication and maintenance of the viral genome
- **Late phase** includes capsid protein production followed by virion assembly, packaging, and release (Graham 2017).

Early phase- Virus entry

Initiation of the virus life cycle begins when the virus particle gains access to the stem cell-like, actively-dividing cells of the basal layer of the epidermis (Egawa 2003, Pyeon, Pearce et al. 2009), through micro-traumas or micro-abrasions of the skin (Doorbar 2005). It has been shown that different HPV types employ different modes of entry, depending upon the type of the cells. For instance, HPV-31 entry is mediated by a caveolin-dependent process (Smith, Campos et al. 2007), while HPV-16 entry is mediated by a clathrin- and caveolin-independent pathway (Spoden, Freitag et al. 2008, Spoden, Kuhling et al. 2013). Thus, so far, no generalized mechanism for the mode of entry has been observed.

The L1 and L2 capsid proteins, encoded by the late region of the viral genome, play a very crucial role, not only in packaging the newly synthesized virions, but also in establishing the viral infection. Following entry, the L1 protein mediates the binding of the virus to the primary cellular receptor, heparin sulphate proteoglycans (HSPGs), and to the extracellular matrix (ECM) through laminin-322 (Schiller, Day et al. 2010). This binding leads to a cyclophilin B-mediated conformational change in the viral capsid structure that exposes the amino-terminus of the L2 capsid protein on the surface of the virion (Bienkowska-Haba, Patel et al. 2009). The L2 amino-terminus then undergoes furin- and/or PC5/6-mediated cleavage, which allows further binding to the secondary receptor present on the plasma membrane of the target cell and which allows the virus particle to be internalized via endocytosis (Richards, Lowy et al. 2006, Kines, Thompson et al. 2009). Once endocytosed, the HPV viral particle is exposed to low pH and various proteases, which aid in viral capsid disassembly. Viral particles are then transported to the multi-vesicular endosomes, facilitated by the ESCRT-1 complex TSG101 and VPS4 proteins, together with the ESCRT-1-associated CD63-syntenin-1-ALIX complex (Broniarczyk, Bergant et al. 2014, Grassel, Fast et al. 2016, Broniarczyk, Pim et al. 2017). At this stage, cyclophilins separate L1 and L2; most of the L1 protein is then subject to lysosomal degradation, while the L2 is exposed to the cytosol (Bienkowska-Haba, Williams et al. 2012). This allows L2 to interact with a variety of cell sorting factors, including the sortin nexin and Vps families of proteins, allowing the L2/DNA complex to avoid lysosomal-mediated degradation, instead being trafficked along the endosomal tubules towards the trans-Golgi network (TGN) (McNally, Faulkner et al. 2017, Siddiqa, Massimi et al. 2018). Eventually, the virus L2/DNA complex enters the nucleus during the breakdown of the nuclear membrane during mitosis (Aydin, Villalonga-Planells et al. 2017). Subsequently, the L2/DNA complex becomes associated with the promyelocytic leukaemia nuclear (PML) bodies, where the virus initiates its genome transcription and establishes nuclear infection (Day, Baker et al. 2004) .

Early phase-Viral Genome Maintenance and proliferation

Upon infection and uncoating, the virus begins its genome amplification by initiating the transcription of its early genes.

E1 and E2, the viral replication proteins, have been shown to play a critical role in setting up the initial replication of the viral genome. The E1 protein recognizes and binds to the viral origin of replication (ori), a palindromic motif in the long non-coding region of the viral genome, and acts as a helicase for the virus replication (Ozbun 2002). The E2 protein possesses a flexible hinge region linking its protein binding domain and DNA binding domain (Hegde 2002). E2 binds to E1 and aids its recruitment to a specific E1-binding motif in the viral origin of replication; this further facilitates the binding of the cellular DNA replication machinery, essential for the viral DNA replication, including DNA polymerase α and replication protein-A (Sanders and Stenlund 1998, Sanders and Stenlund 2000, McBride 2013). Studies performed in cell lines containing episomal HPV genomes suggest that, at this stage, the viral episomes are maintained at a low copy number; proposed to be approximately 50 to 100 copies per cell, but which may vary from lesion to lesion and also depend on the site of infection (Maglennon, McIntosh et al. 2011, Doorbar, Egawa et al. 2015). This low copy number is known to be maintained by the E8^E2 protein, using the cellular NCoR/SMRT complex, constituting the initial phase of the viral genome amplification and maintenance (Dreer, van de Poel et al. 2017). After replication, the E2 protein interacts with the Brd4 protein and tethers the HPV genomes to the cellular chromosomes, allowing the equal partitioning of the viral genome into the daughter cells, together with the host cell DNA (Iftner, Haedicke-Jarboui et al. 2017).

Studies have suggested that viral proteins are expressed at low levels in the infected lower basal cells (Peh, Middleton et al. 2002), to avoid activating the local immune response (Westrich, Warren et al. 2017), which otherwise might lead to clearance of virus particles. This is known to be achieved by the E2 protein, which helps in the DNA replication and transcriptionally regulates the P97 promoter, controlling the expression of the viral genes E6 and E7 (Smith, White et al. 2010, Smith, Haberstroh et al. 2014). In this way, HPV successfully maintains the infection in the epithelial cells for a significant period of time. These infected cells carry the viral genome with them as they differentiate and move towards the upper layers of epithelium (Oldak, Smola et al. 2004, Doorbar 2005).

The increased proliferation of the parabasal epithelium layer is linked to the increased expression of the two HPV oncogenes, encoding the E6 and E7 proteins. These proteins are transcribed from the early promoter of the viral genome (p97 in HPV-16 and p105 in HPV-18). Regardless of their role as oncogenes, it has been shown that their expression is essential for the productive HPV viral life cycle (Graham 2010).

During early infection, the expression of E7 protein overrides the checkpoint at the end of the G1 phase of the cell cycle to promote S-phase entry in epithelial cells that would normally undergo terminal differentiation, thus increasing the pool of the cells that are active in DNA replication (Roman and Munger 2013). This is considered to be one of the most critical steps in achieving viral genome replication in the terminally differentiated cells from the mid-to-upper epithelial layers that would, in a normal scenario, exit the cell cycle. Differentiation in these cells is delayed, and the cells are maintained in a pseudo-S phase, where the viral genome replication occurs. The mechanism by which HPVs targets and disrupts the cell cycle control is well studied. E7 targets and degrades the retinoblastoma protein (pRb), as well as the other pocket proteins, p107 and p130, which generally bind and sequester the E2F transcription factors. Degradation of pRb, p107 and p130 results in a pool of free E2F that is then available to activate further the transcription of several cell-cycle regulatory proteins, such as cyclin E and cyclin A, thereby stimulating the G1 to S-phase transition and helping to promote cell cycle progression and transcription (Helt and Galloway 2001, Hwang, Lee et al. 2002, Zhang, Chen et al. 2006, Roman and Munger 2013).

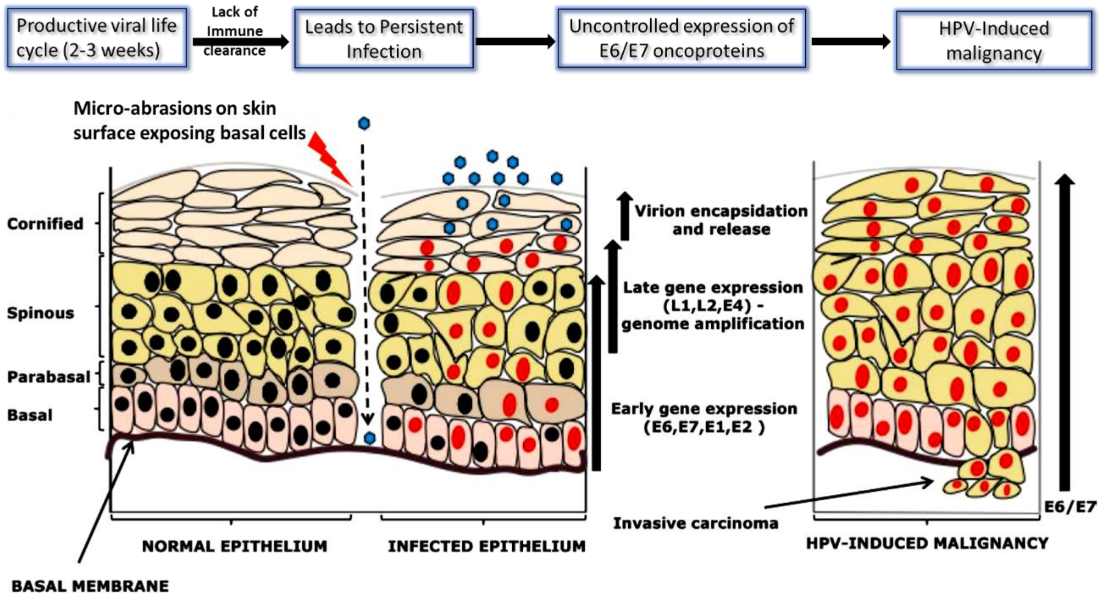
In the normal scenario, cells respond to the unprogrammed cell proliferation by inducing apoptosis, so the above-mentioned E7 activities would probably cause the activation of apoptotic pathways, resulting in cell death. To overcome this possibility, HPVs express the E6 protein, which targets the p53 tumor suppressor, through two distinct pathways (Vande Pol and Klingelhutz 2013, Mittal and Banks 2017). The first of these is the direct ubiquitination of p53, with the help of the E6AP ubiquitin ligase, which leads to p53 degradation through the proteasome. The second is the repression of p53 transcriptional activity by targeting its co-activator CBP/p300 (Patel, Huang et al. 1999, Vande Pol and Klingelhutz 2013).

The decrease in the levels of p53 protein in the basal cells contributes to perturbed p53 transcriptional activity, one of the consequences of which is a reduction in the levels of

Notch receptor present on the cell surface. Since Notch signaling plays an essential role in regulating basal cell density and commitment to differentiation, HPV-induced reduction in Notch activity thereby provides the infected cell with a competitive advantage over its neighbouring uninfected cells, thus favoring the expansion in the population of infected basal cells (Woodworth, Cheng et al. 1992, Boxman, Mulder et al. 2001).

Late phase: Viral packaging and release

The final stage of the productive viral life cycle is the packaging of the viral genome into the infectious particle, which involves the expression of both structural proteins: the major capsid protein L1 and minor capsid L2 protein. Nuclear localization signals present in both L1 and L2, mediate the translocation of the proteins to the cell nucleus, where the assembly of the virion particles takes place (Zhou, Doorbar et al. 1991, Darshan, Lucchi et al. 2004, Buck, Day et al. 2013). For almost all the high-risk HPV types, the expression and the nuclear localization of L2 appears to precede L1 expression, and the assembly of the infectious virion particles also requires the activity of E2 (Doorbar and Gallimore 1987, Day, Roden et al. 1998, Florin, Sapp et al. 2002). It has been shown that the L2 protein recruits L1 to the PML bodies in the nucleus, thus enhancing the packaging ability of the capsid proteins (Zhou, Stenzel et al. 1993, Stauffer, Raj et al. 1998). HPV L2 protein is known to form a direct complex with the nascent viral DNA and aid in its the nuclear accumulation (Wang and Roden 2013). Once the viral particles are formed, the E1^E4 protein helps the virus to survive in the harsh extracellular environment by forming amyloid fibers with the help of transglutaminase 3. These amyloid fibers help in disrupting the keratin structures and the normal cornified layer of the upper epithelium cells, thereby supporting the dispersal of the new virions (Brown, Kitchin et al. 2006, McIntosh, Martin et al. 2008, McIntosh, Laskey et al. 2010).



Modified from **(Tomaic 2016)**

Figure 2. Schematic diagram showing HPV life cycle: HPV infects the basal layer of the epidermis, gaining access through micro-abrasions on the skin surface. As the epithelium differentiates, all the viral genes are expressed in a coordinated manner, with E6/E7 triggering expansion of pseudo-S-phase-competent cells. This, in turn, facilitates amplification of the viral genome, completes its life cycle and eventually leads to the release of the new virion progeny from the uppermost layer of the differentiated epithelium. Failure of the immune system to clear the leads to persistent infection, which pre-disposes the host cells towards HPV-induced malignancy, in which the virus fails to complete its productive life cycle, while the E6 and E7 proteins are expressed in an uncontrolled manner.

Life-cycle deregulation and cancer progression

In the normal virus life cycle the virus modifies the normal differentiation process of the lower epithelium, resulting in the formation of low-grade squamous intraepithelial lesions (LSIL), clinically manifested as cervical intra-epithelial neoplasia grade 1 (CIN1). It is thought that CIN1 lesions represent a transient HPV infection, with a low possibility of progressing to cervical cancer (Martin and O'Leary 2011, Doorbar, Egawa et al. 2015). At this stage, the expression levels of both HPV E6 and E7 proteins are so low that they do not compromise the regular functioning of the cellular proteins sufficiently to initiate cancer progression (Doorbar, Quint et al. 2012). Infection with multiple HPV types is very common during CIN1 (Martin and O'Leary 2011), but these are eventually cleared by the host immune system over a period of several months, leading to the regression of approximately 80%-90% of CIN1 cases, as shown in Figure 3 (Cuschieri, Cubie et al. 2004).

However, if the viral infection evades the host immune response, or if the host is immune-suppressed, then the cells can remain persistently infected for many years. In such cases, the infected tissues can progress to high-grade squamous intraepithelial lesions (HSIL), clinically manifested as CIN2 and CIN3, having the potential to progress to carcinoma *in situ*, that can further penetrate the basement membrane barrier, leading to invasive carcinoma (McMurray, Nguyen et al. 2001). Continuous expression of the high-risk HPV E6 and E7 oncoproteins predisposes the cell to the accumulation of genetic alterations, which increasingly contribute to the progression of malignancy, eventually leading to metastatic cancer (Graham 2017).

In approximately 70% of high-grade lesions, the viral episome is thought to integrate into the host cell chromosome. Integration typically results in the increased expression and stability of transcripts encoding the viral oncogenes E6 and E7, which are known to inactivate and/or accelerate the degradation of numerous cellular proteins, in turn leading to accumulation of genetic changes (Jeon and Lambert 1995, Munger, Baldwin et al. 2004). This provides a selective advantage for the clonal expansion of cells with an integrated HPV genome over the uninfected cells, thereby contributing towards the progression of malignancy (Schwarz, Freese et al. 1985, Shirasawa, Tomita et al. 1989, Jeon and Lambert 1995).

Several genome-wide studies have identified a few loci for HPV genomic integration that are highly correlated with transcriptionally active regions of the genome and the common fragile sites (CFS) (Thorland, Myers et al. 2000, Christiansen, Sandve et al. 2015). The suggested CFSs include FRA13C (13q22), FRA3B (3p14.2) and FRA17B (17q23) (Thorland, Myers et al. 2003). This integration disrupts the HPV regulatory E2 protein, resulting in a loss of negative feedback control of both E6 and E7 oncogene expression. Therefore, it has been considered a crucial event in the pathogenesis of cervical neoplasia, as it provides a growth advantage to the HPV-integrated cells (Romanczuk, Thierry et al. 1990, Badaracco, Venuti et al. 2002, Bechtold, Beard et al. 2003). However, the mechanism behind integration of the HPV genome into the host cell chromosome is poorly understood. Several reports have suggested that integration occurs in response to DNA damage. Winder et al in 2007, demonstrated that depletion of Ku70 generated double-stranded DNA breaks (DSBs) in W12 cells, which stably contain an HPV 16 episome, and led to *de novo* viral integration events, with the consequent loss of the viral episome (Winder, Pett et al. 2007). Apart from this, it has been shown that patients with cervical cancer have markedly reduced levels of DNA-dependent protein kinase (DNA-PK), which is known to be important in repairing cellular DSBs (Someya, Sakata et al. 2006). These studies implicate the involvement of DSBs with HPV episome loss and viral genome integration in cervical cancer. Persistent HPV viral infection may also promote viral integration by activating the transcription of E6 and E7 oncogenes, which in turn increases the level of intracellular ROS (Reactive Oxygen Species)/RONS (Reactive Oxygen Nitrogen Species), leading to DNA damage in human keratinocytes (Kessis, Connolly et al. 1996, Williams, Filippova et al. 2014, Chen Wongworawat, Filippova et al. 2016). Elevated levels of oxidative DNA damage coincide with increased HPV infection, viral-host integration, and dysplastic cervical lesions (Visalli, Riso et al. 2016). High levels of E1 have been shown to induce over-replication of the viral DNA, generating onion skin structure heterogenous replicational intermediates that in turn may endanger the genomic integrity and eventually lead to integration of the viral genome (Mannik, Runkorg et al. 2002, Kadaja, Isok-Paas et al. 2009). Another possible means of promoting inadvertent integration could be as a result of the E2-Brd4 complex, which associates with regions of host chromatin that are susceptible to replication stress, and replication foci frequently form close to common fragile sites (Jang, Shen et al. 2014, McBride, Warburton et al. 2021).Thus, several events are likely to contribute towards the HPV integration events and its subsequent evolution, aborting the viral genome amplification and strongly driving carcinogenesis.

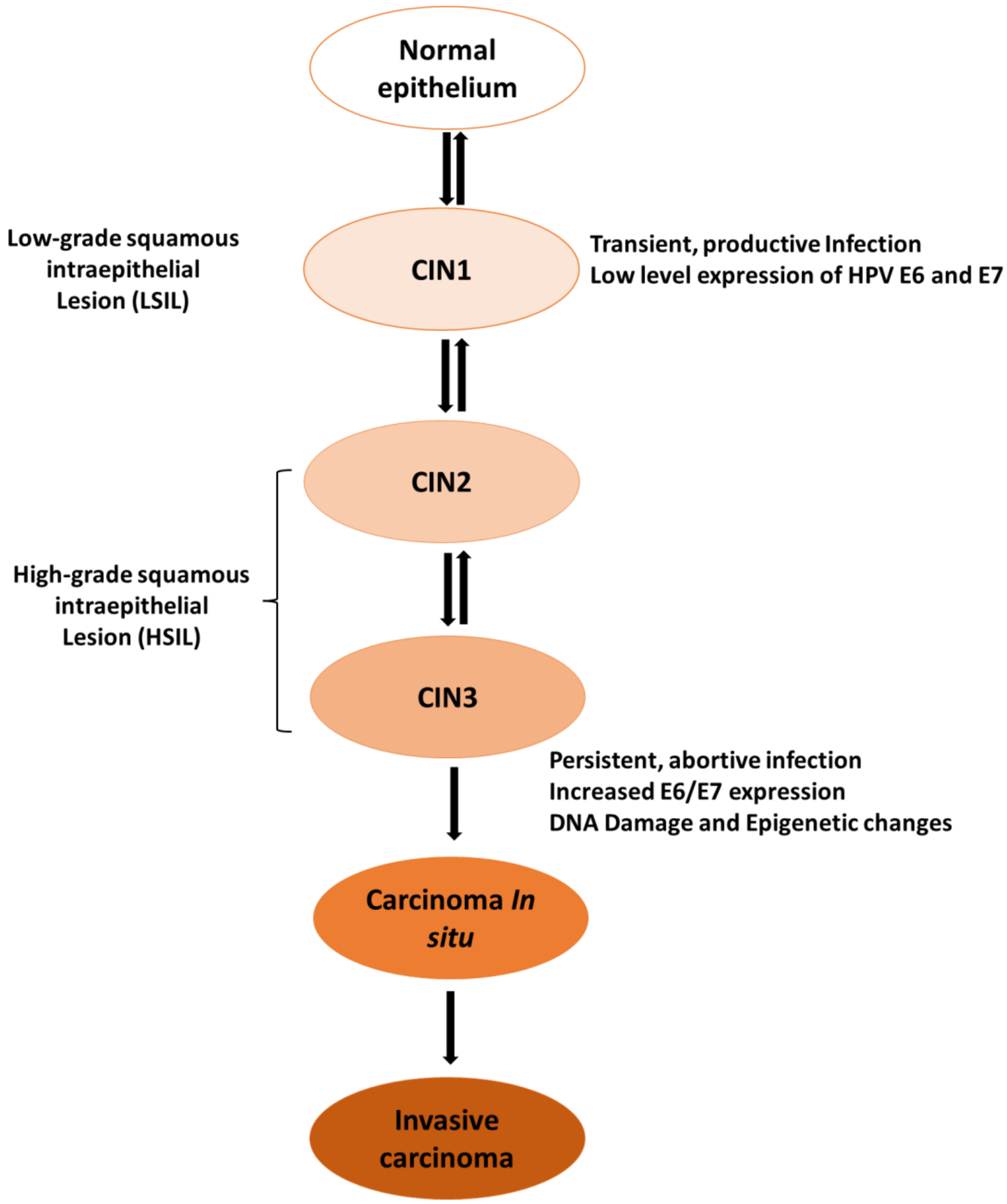


Figure 3. Schematic representation of cervical cancer malignant progression: HPV infection in the basal layer of the epithelium leads to CIN1 lesions, in which the virus undergoes an ordered pattern of viral gene expression leading to new viral particle synthesis and release from the upper epithelial layers. The infection at this stage is transient and in approximately 90% of the cases, the infection is cleared and the lesion regresses back to normal cells. CIN2 is known to be a mixture of CIN1 and CIN3, which can further progress towards CIN3. Persistent viral infection and increased expression of HPV E6 and E7 proteins result in the accumulation of epigenetic

alterations and increased DNA damage, leading to the integration of the genome into the host chromosome and progression to cancer.

HPV oncoproteins

A plethora of studies over the period of many decades have provided insights into the HPV E6 and E7 oncoproteins as key players in HPV-induced malignant cancer progression. E6 and E7 are known to be the only viral genes that are always maintained and expressed in HPV-positive cervical cancer lesions (Durst, Gissmann et al. 1983, Schwarz, Freese et al. 1985). Continued expression of HPV E6 and E7 oncogenes is an absolute requirement for the growth and progression of HPV-positive cervical cancer cells *in vitro* as well as *in vivo* (Roman and Munger 2013, Vande Pol and Klingelhutz 2013), and inhibition of E6/E7 induces cellular senescence, leading to cell death (Yamato, Yamada et al. 2008, Jabbar, Abrams et al. 2009). Collectively, these data indicate that the HPV-positive cervical cancer cells are "E6/E7 oncogene-addicted" and they together contribute to promoting many of the main phenotypic changes of a typical cancer cell that have been described as "hallmarks of cancer" (Hanahan and Weinberg 2011), namely, sustained proliferative signaling, tissue invasion and metastasis, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and resisting cell death (Mesri, Feitelson et al. 2014).

E7 oncoprotein

HPV E7, is a small protein of approximately 100 amino acid residues and was the first HPV oncoprotein to be discovered. Structurally, E7 consists of 3 conserved domains; CD1, CD2 and CD3 present in the N-terminal half of the protein; having sequence homology to adenovirus E1A and SV40 Large T antigen. The C-terminal end consists of a zinc-binding domain containing two Cys-X-X-Cys motifs separated by a stretch of 29 amino acid residues, and this acts as a dimerization/multimerization domain, as shown in Figure 4 (Munger, Baldwin et al. 2004) . Serines positioned at 31 and 32 in HPV E7 oncoprotein, are considered to be potential sites of phosphorylation by casein kinase II (CKII) (Firzlaff, Galloway et al. 1989, Barbosa, Edmonds et al. 1990). Apart from S31 and S32, another Serine residue positioned at 71 in the C terminus has also been reported to be phosphorylated, but the kinase involved has not yet been identified (Massimi and Banks 2000). It has been recently reported that the CKII phosphorylation site plays an essential role in maintaining the functional integrity of the HPV E7 oncoprotein and also in maintaining the transformed phenotype of the cervical cancer-derived cell lines (Basukala, Mittal et al. 2019, Basukala, Sarabia-Vega et al. 2020). Post-transcriptionally HPV 16E7 is also regulated by the proteasome system, where it interacts with Cullin 1, an SCF (Skp-Cullin-F box) ubiquitin ligase, which mediates the ubiquitination of HPV E7 (Oh, Kalinina et al. 2004). Interestingly, the ubiquitination of E7 oncoprotein requires a free N-terminus, as it has been shown that blocking the N-terminus and not the C-terminus of E7 inhibits ubiquitin conjugation and subsequent degradation (Reinstein, Scheffner et al. 2000).

The conserved domain 1 (CD1) of E7 is 20 amino acids long and is known to be critical for inducing cell cycle progression to S-phase and cellular transformation (Banks, Edmonds et al. 1990, Brokaw, Yee et al. 1994, Demers, Espling et al. 1996). E7, by using this domain, binds directly to p600, which is thought to be essential for HPV-16 E7-mediated cell transformation independently of the interaction with pRb (DeMasi, Huh et al. 2005, Huh, DeMasi et al. 2005); however the exact role of p600 in E7's activities is not yet fully understood. However, it would appear to play an essential role in the ability of E7 to bring about cell transformation as loss of interaction, such as that obtained following N-terminal tagging of E7, also results in a loss of transforming activity. Furthermore, p600 acts as a ubiquitin ligase, and E7 uses this interaction to promote degradation of PTPN14 proteins (White, Munger et al. 2016, Szalmas, Tomaic et al. 2017). Interestingly, the E7 proteins of other HPV types, including those that are not associated with cancer, also interact with the p600 complex, suggesting a role in viral replication (White, Kramer et al. 2012). In addition to p600, CD1 also binds to p300/CBP-associated factor (P/CAF). This interaction downregulates the activation of NF-kβ family proteins, which, in turn, represses the activation of the IL-8 promoter, thus assisting the virus to evade the host immune surveillance (Huang and McCance 2002).

Next, the CD2 region of E7 (ranging in length from 20-38 amino acid residues) mainly constitutes the CKII phosphorylation site and the LXCXE binding motif (Barbosa, Edmonds et al. 1990). E7 is highly multifunctional in nature and targets many cellular proteins and the most important of these is the retinoblastoma tumour suppressor protein (pRB). All high-risk HPV E7 proteins bind directly to pRB through the LXCXE motif. This interaction, furthermore, recruits cullin 2 ubiquitin ligase and promotes the proteasome-mediated degradation of pRB (Imai, Matsushima et al. 1991, Boyer, Wazer et al. 1996, Huh, Zhou et al. 2007). As a result, this causes the dissociation of E2F from the pRB/E2F transcriptional repressor complex, increases the transcriptional activation of E2F-responsive promoters, and stimulates the transition of cells from G0 to G1, and then into S phase of the cell cycle (Davies, Hicks et al. 1993). In addition, HPV-16 E7 has been shown to target several members of the E2F transcription factor family, including the transcriptional activator, E2F1 and the transcriptional repressor E2F6. HPV E7 interacts with E2F6 and restrains it from performing its function as a transcriptional repressor in opposition to the transcriptional activation of E2F1. By this interaction HPV can counteract the up-regulation of E2F6 that takes place as a result of E7-mediated activation of E2F1. Besides this, E2F6 also has role in maintaining cells in a quiescent state and its de-regulation could be an important strategy of the HPV E7 to allow cells that are committed to exit the cell cycle and differentiation to remain in a S-phase-competent state, enabling them to initiate growth and proliferative signalling (Trimarchi, Fairchild et al. 1998, McLaughlin-Drubin, Huh et al. 2008). E7 also binds to the other pRB-related pocket proteins, p107 and p130. This binding plays an important role in controlling the proliferation, differentiation and apoptosis of the cells through their interactions with other downstream molecules (Morris and Dyson 2001) and in the case of p130 proteasomal degradation facilitates cell cycle deregulation and impairment of cellular differentiation in HPV 16 and 18 E7 expressing cells (Gandhi, Nor Rashid et al. 2021)

The C-terminal part of HPV E7 contains the conserved domain 3 (CD3), a 38-98 amino acid domain that negatively regulates several cyclin-dependent kinases (CDKs), thus further contributing to cell cycle dysregulation by overcoming the DNA damage-induced cell cycle arrest. CDKs are the family of kinases that drive the cell cycle progression and are regulated by cyclin-dependent kinase inhibitors (CKIs). It has been previously reported that the regulatory subunits of CDK2, cyclin A and cyclin E, are expressed abundantly in HPV E7 expressing cells. Mechanistically, this is regulated by E7 binding to the CKIs, $p21^{CIP1}$ and $p27^{KIP1}$ (negative regulators of cyclin A and cyclin E activities), through its carboxy terminus domain, consequently nullifying the inhibitory effects on cyclin A and cyclin E-associated activities. Intriguingly, both p21 and p27 have been shown to be involved in TGF-β-mediated growth inhibition (Elbendary, Berchuck et al. 1994, Polyak, Kato et al. 1994, Datto, Li et al. 1995). Thus, HPV E7-mediated inactivation of both p21 and p27 may contribute to the escape of

HPV-infected cells from TGF-β-mediated growth arrest. Low-risk HPV E7 also binds to p21, but with a much lower affinity and decreased ability to counteract the inhibitory activity of p21. However, both high-risk and low-risk type HPV E7 can bind to cdk and/or to cyclin subunits either directly, or indirectly through pRB, p107 and p130, allowing the maintenance of consistent cdk2 activity in the cells (He, Staples et al. 2003, Nguyen and Munger 2008). Interestingly, E7 is also shown to promote cdk2 activation by increasing the expression levels of CDC25A phosphatase, which induces the tyrosine dephosphorylation of cdk2 (Katich, Zerfass-Thome et al. 2001, Nguyen, Westbrook et al. 2002). Thus, the ability of HPV E7 to override CKIs and disrupt pRB/E2F complexes, resulting in increased levels of cyclin A and E, creates a replication-competent cellular environment in the differentiating keratinocytes through deregulation of the G1/S cell cycle checkpoint and enhancing proliferation.

Expression of high-risk HPV E7 also induces mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle, thereby generating abnormal centrosome duplication (Duensing, Lee et al. 2000). This occurs through a mechanism independent of its potential to inactivate pRB family of proteins but involves interaction with the centrosomal regulator, γ-tubulin (Duensing and Munger 2003, Nguyen, Eichwald et al. 2007). Taken together, these studies suggests that E7 disrupts the centrosome homeostasis which contributes directly towards genomic instability and cancer progression.

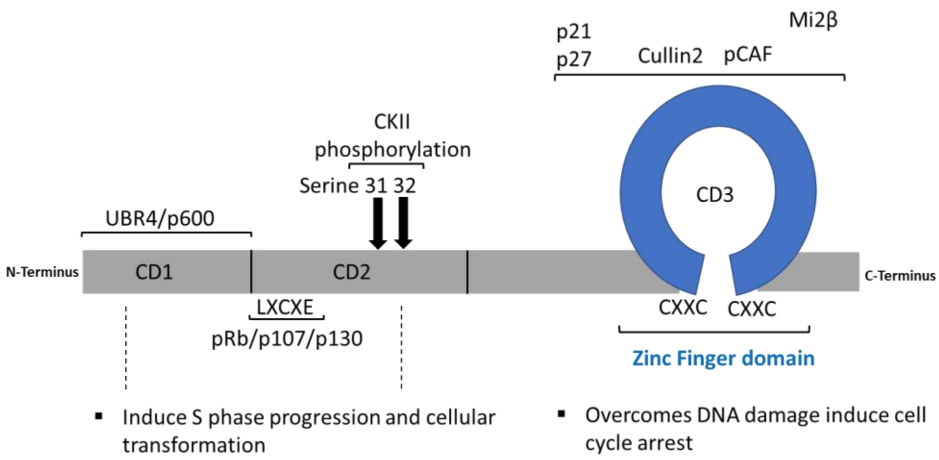


Figure 4. Schematic diagram of HPV E7: HPVs E7 structurally consists of the conserved regions CD1, CD2 and CD3, having sequence homology with the adenovirus E1A protein and SV40 T antigen. These conserved regions are important for maintaining the protein function and integrity. The Zinc finger (in blue) is also shown, together with LXCXE motif that binds to the tumour suppressor Rb and the serine residues 31 and 32 that are known CKII phosphorylation sites.

HPV E6 oncoprotein

HPV E6 is a small protein of approximately 150-160 amino acid residues. As shown in Figure 5, structurally, HPV E6 contains two zinc fingers, separated by a central domain of 35 amino acids. The zinc fingers domains present at both N and C-terminus are formed by two pairs of CXXC motifs. The zinc fingers are involved in the interaction with numerous cellular proteins. In the E6 proteins from high-risk HPV types there is a short Class 1 PDZ-binding motif (PBM) located at the extreme C-terminus which includes the XS/TXV/L sub-motif. This motif allows E6 to recognize and bind to PDZ domain-containing proteins. Altogether, these domains permit high-risk HPV E6 oncoprotein to target a myriad of cellular proteins (described in detail in later sections) and thus, impose its influence on several cellular pathways, giving an advantage for viral genome amplification while, in some cases, inadvertently contributing to HPV-induced carcinogenesis (Wallace and Galloway 2015, Tomaic 2016).

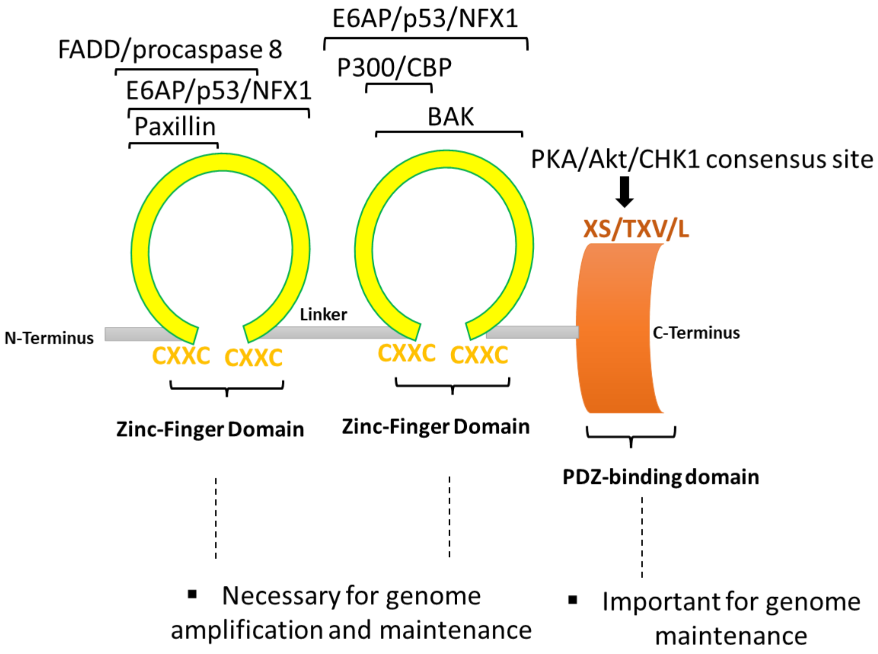


Figure 5. Schematic diagram of HPV E6: HPV E6s contain two zinc finger domains (shown in yellow) connected by a short linker sequence. The Zinc fingers are involved in binding to several cellular proteins, as shown. The C-terminus of high-risk HPV E6s contains a PDZ-binding motif, that also contains a potential consensus sequence for PKA, Akt or CHK1 phosphorylation.

Key molecular targets of HPV E6

HPV E6 and the Ubiquitin proteasome system (UPS)

In eukaryotic cells, proteins normally exist in a very dynamic state, that is they undergo constitutive degradation and replacement with newly synthesized proteins. Ubiquitination is an enzymatic process in which a ubiquitin protein is attached to a target protein. This process involves binding of the ubiquitin's last amino acid (glycine 76) to a lysine present on the target substrate. As a result, an isopeptide bond is formed between the ubiquitin's glycine carboxyl group and the amino group of the target protein's lysine (Pickart 2001). However, degradation (either via the proteasome or the lysosome) is not the only fate of the ubiquitinated protein; ubiquitination also affects other cellular processes such as coordinating the cellular localisation of the proteins, modulating protein-protein interactions and regulating the activation of proteins. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and an

N-terminal methionine (M1) residue, that serve as points of ubiquitination. Cellular substrates are ubiquitinated with a polymer of ubiquitin (polyubiquitination) are selectively targeted to 26S proteasome, whilst those ubiquitinated with one ubiquitin (monoubiquitination) are targeted for endocytosis and eventually degraded in the lysosome (Glickman and Ciechanover 2002, Schnell and Hicke 2003, Mukhopadhyay and Riezman 2007, Komander 2009). Monoubiquitination is also known to affect other cellular processes involving endocytosis, chromatin regulation, protein sorting and trafficking (Miranda and Sorkin 2007, Ikeda and Dikic 2008). Within polyubiquitin chains, ubiquitin can form eight different linkage types, using one or more combinations of the seven internal lysine residues (K6, K11, K27, K29, K33, K48, K63) or methionine at position 1 (M1). The K48-linked ubiquitin chain are the predominant linkage type that triggers the proteasome- dependent degradation of the target substrates (Hicke 2001). In contrast, the second most abundant form of ubiquitin chain, K63-linked is primarily known for non-proteolytic signalling, such as DNA damage response, cell trafficking, autophagy and immune responses (Nathan, Kim et al. 2013, Akutsu, Dikic et al. 2016). Ubiquitin has also been shown to play a crucial role in the first steps of some forms of viral replication. For example, treatment with proteasome inhibitors have been reported to inhibit herpes simplex virus (HSV) entry at an early step, immediately after the penetration of the viral capsid into the host cell (Delboy, Roller et al. 2008, Delboy and Nicola 2011). The Kaposi Sarcoma associated herpesvirus (KSHV) (Greene, Zhang et al. 2012), the influenza virus (Chen and Zhuang 2008) and adenoviruses (Wodrich, Henaff et al. 2010) are additional examples of viruses which are dependent upon the UPS for their entry into target host cells.

Degradation of proteins by the UPS is highly complex and follows a hierarchy involving two successive steps that are tightly regulated by the cell. The first is the attachment of several ubiquitin molecules onto the target substrate, which is usually carried out by three different enzymes acting sequentially, namely; E1; the ubiquitin-activating enzyme; E2; the ubiquitin-conjugating enzyme and a substrate-specific E3; the ubiquitin-protein ligase (Glickman and Ciechanover 2002). The second step is the degradation of the ubiquitin-conjugated target substrate by the 26S proteasome complex, which breaks them into small peptides and also releases free ubiquitin. The released peptides and ubiquitin are recycled to replenish the cellular pools of building blocks and are used to generate new proteins, to be followed, yet again, by their degradation as shown in Figure 6 (Glickman and Ciechanover 2002).

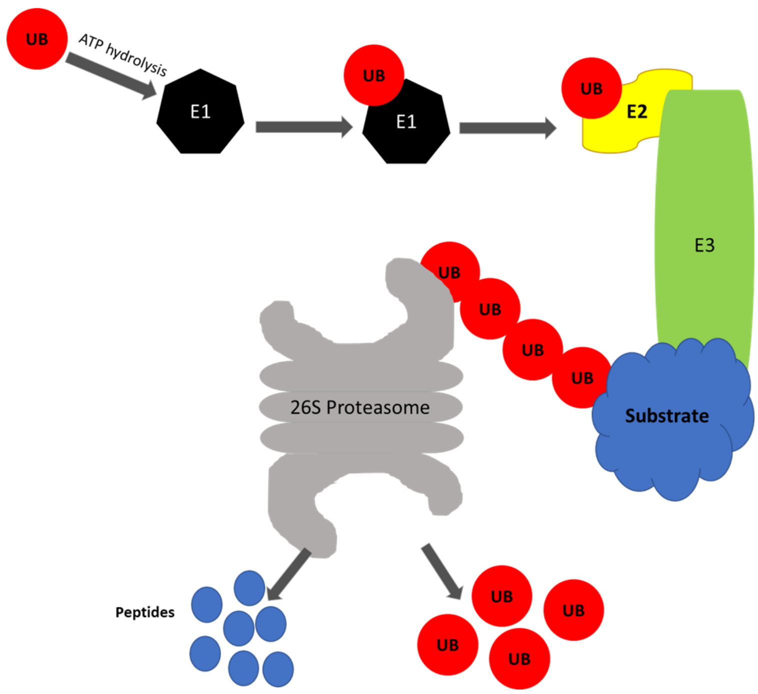


Figure 6. Schematic diagram of the Ubiquitin proteasome system: The Ubiquitin proteasome system involves a cascade of steps. To begin with, E1 activates the ubiquitin in an ATP-dependent manner. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme, E2. E2 then transfers the activated ubiquitin moiety to the target substrate that is bound specifically to a unique ubiquitin ligase, E3. Successive conjugation of ubiquitin moieties to one another generates a polyubiquitin chain. The polyubiquitin chain serves as a binding and degradation signal for the 26S proteasome. The substrate is degraded to short peptides, followed by a release of free and reusable ubiquitin molecules.

The E1 ubiquitin-activating enzymes catalyse the initial activation of ubiquitin, binding ubiquitin to its active cysteine residue in an ATP-dependent manner. It has been shown that the rate of transfer of ubiquitin from E1 is independent of the type of recipient E2 enzyme (Schulman and Harper 2009).

The E2 ubiquitin-conjugating enzymes receive the activated ubiquitin from E1 via a transthiolation reaction. In the human genome approximately 40 different E2 enzymes have so far been identified. They are known to act as central players in the trio of this enzymatic reaction cascade responsible for ubiquitination of the target proteins (Stewart, Ritterhoff et al. 2016, Zheng and Shabek 2017).

Substrate-specific E3 ubiquitin-protein ligases recognize the diverse target proteins and facilitate the covalent linkage between ubiquitin moieties and the target substrate. More than 600 different E3 ligases have been identified in mammalian cells, providing them with the ability to ubiquitinylate many different target proteins with high specificity. E3 ubiquitin-protein ligases are broadly sub-divided into 2 categories, depending on their homology domains; the RING (really interesting new gene) type E3 ligases and HECT (homologous to E6-AP C-terminus). The RING type are known for their U-box fold catalytic domain, which facilitates the direct transfer of ubiquitin from E2 to the target substrate. The best-described RING type E3 ligases are from the cullin RING ligase (CRL) superfamily, which includes the CRL1, also known as SKp1-Cul1-F-box protein complex, and the APC/C (anaphase promoting complex/cyclosome) complex. In the case of HECT type E3 ligases, the Ub is first transferred from the E2 to E3, which involves the transfer of ubiquitin from E2-ubiquitin thioester to an internal conserved active cysteine present in the C-terminus of the HECT domain, forming an E3-ubiquitin thioester intermediate. Simultaneously, other domains of the HECT E3 ligases recruit the target proteins, followed by subsequent handover of ubiquitin from the intermediate E3 thioester to a lysine residue on the target protein, as shown in Figure 7 (Zheng and Shabek 2017).

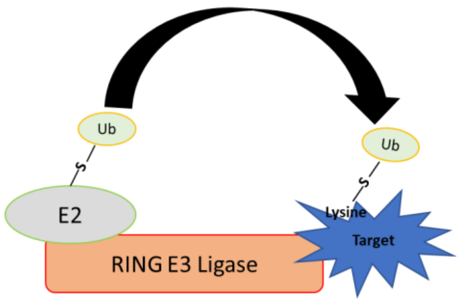


Figure 7: Schematic diagram of different E3 ligases: The E3 ubiquitin-protein ligases are classified into two ubiquitin-ligase groups based on differences in domain structure: RING type and HECT type. RING-type E3s add Ub moieties directly from E2 to the target substrate, functioning as a scaffold, whereas in the case of HECT type E3 ligases, Ub residues first bind with a cysteine residue on the E3 to form an

intermediate that forms thioester bonds with ubiquitin and then transfers the Ub to the target substrate.

The Ubiquitin proteasome system is known to be responsible for maintaining the turnover of many cellular proteins and tumour suppressors that, if not correctly removed from the cells, might lead to cellular or DNA damage and, potentially, to malignancy. Many DNA tumour viruses, such as Adenovirus, HPV and SV40, have evolved to exploit the host cell UPS for their own benefit: either degrading tumour suppressor proteins or regulating their expression so as to maximize the viral replication. The most common strategy that all these viruses employ is their potential to target the two major tumour suppressors p53 and pRb, which are known to regulate many cellular pathways involved in controlling tumour progression (Blanchette and Branton 2009). For instance, Adenovirus encodes two viral proteins, E1B-55K and E4-ORF6, EBV encodes BZFL1 protein, KSHV encodes LANA protein and HPV encodes E6 protein, all of which have been shown to form complexes with ubiquitin proteasome system to target p53 for degradation (reviewed in Adhya and Basu 2010) . Thus, this makes tumour viruses very useful tools for identifying the key cellular control points, having had millions of years of evolution to make sure that they have the maximum influence for the minimum effort.

HPV was the first small DNA tumour virus discovered to use the host cell ubiquitin proteasome pathway for its own purposes: directing the degradation of the most studied tumour suppressor, p53. This degradation of p53 by HPV E6 oncoprotein is mediated by a cellular protein, E6-associated protein (E6-AP) (Scheffner, Werness et al. 1990, Band, De Caprio et al. 1991, Scheffner, Takahashi et al. 1992). It was found that E6 and p53 associate and form a complex with E6-AP in order to degrade either wildtype or mutant forms of p53 (Scheffner, Takahashi et al. 1992). In a normal scenario, the activity of E6AP ubiquitin ligases is strictly regulated, however its loss of expression has been reported to cause a neurodevelopmental disorder, known as Angelman syndrome (Tomaic and Banks 2015), whereas its increased expression has been shown to be associated with autism spectrum disorders (Samaco, Hogart et al. 2005).

E6-E6AP together function as an E3 ligase in conjugation with E2-conjugating enzymes UbcH5 (Scheffner, Huibregtse et al. 1993, Scheffner, Huibregtse et al. 1994), UbcH6 (Nuber, Schwarz et al. 1996) and UbcH7 (Blumenfeld, Gonen et al. 1994, Ciechanover, Shkedy et al. 1994, Nuber, Schwarz et al. 1996) to target p53 for proteasomal degradation. In fact, E6AP is recognised as the founding member of the HECT type E3 family of ligases (Huibregtse, Scheffner et al. 1995), since it was the first E3 ligase that was found to form an intermediate ubiquitin thioester bond before attaching ubiquitin moieties to its target substrate (Scheffner, Nuber et al. 1995). The HECT domain at the C-terminus of the E6AP is composed of approximately 350 amino acid residues, with the last 32-34 amino-acid sequence containing the conserved cysteine residue that is most likely involved in the formation of ubiquitin thioester bond (Scheffner, Nuber et al. 1995).

E6 is also known to associate with HERC2, a HECT-domain E3 ubiquitin ligase, containing NEURL4 and mitogen-activated protein kinase-6 (MAPK6) complex through E6AP (Martinez-Noel, Galligan et al. 2012). Another HECT domain-containing E3 ubiquitin ligase, EDD/UBR5 has been shown to interact with HPV-18 E6. Loss of EDD induces the proteolytic activity of E6/E6AP complex to mediate the degradation of its target proteins, particularly p53 (Tomaic, Pim et al. 2011). Additionally, interaction of HR HPV E6 oncoprotein with EDD has been shown to destabilize TIP60, a tumour suppressor, in a proteasome-dependent manner, thus contributing towards the development of HPV-induced carcinogenesis (Subbaiah, Zhang et al. 2016).

Different HPVs E6 from α and β HPV types have been demonstrated to interact with the TRIM25 ubiquitin ligase and USP15, to inhibit the RIG-1-mediated innate immunity response, suggesting that the role of HPVs E6 in terms of modulating the immune surveillance is conserved across different HPV types (Chiang, Pauli et al. 2018). Poirson *et al.*, in a recent study, screened a library of 590 cDNAs related to Ubiquitin Proteasome System (UPS), using the *Gaussia princeps* luciferase complementation assay (GPCA) and identified many ubiquitin ligases as interactors of HPV E6. Results indicated various new target proteins, including three RING-type Ub ligases MGRN1, LNX3 and LNX4 (Thomas and Banks 2015, Poirson, Biquand et al. 2017). Interaction of E6 with LNX3 has been shown to target it for PDZ-dependent, proteasome-mediated degradation, which in turn activates STAT-5β, providing a link between viral life cycle and differentiation-related STAT signalling (Thomas and Banks 2015). Amongst these, MGRN1 was shown to bind with E6 proteins from LR HPV-6 and HR HPV types 16, 18 and 33, as well as β-types HPV types 8 and 38.

Interestingly, interaction with LNX4 was limited to HPV-16 E6, while LNX3 interacted with E6 oncoproteins from HR HPV types 16, 18 and 33 and β-HPV type 8 E6. The other ubiquitin ligases studied were shown to solely interact with HPV-16 E6, that includes ITCH, TRAF6, TRAF5, UBAC1, VHL, XIAP, RNF25 and RNF40, however, the biological consequences of these interactions in the viral life cycle and in HPV-mediated malignancies are not yet known (Poirson, Biquand et al. 2017).

It has been reported that binding of E6 with E6AP induces conformational rearrangements in E6AP structure, which in turn stimulates its E3 ubiquitin ligase activity (Mortensen, Schneider et al. 2015, Sailer, Offensperger et al. 2018). In addition, recently it has been shown that multiple regions of E6AP are involved in its activation and interaction with E6 oncoprotein (Drews, Brimer et al. 2020). Initial *in vitro* experiments unravelling the HPV-16 and -18 E6-mediated ubiquitination of p53 were performed using the rabbit reticulocyte lysate translation system (Scheffner, Werness et al. 1990, Werness, Levine et al. 1990). Subsequent experiments demonstrated that, in addition to E1 and E2 ligases, rabbit reticulocyte lysate contributed one more factor that played a key role in maintaining the stability of E6-p53 complex for p53 degradation (Huibregtse, Scheffner et al. 1991), and this was eventually named E6AP (Huibregtse, Scheffner et al. 1993). Corresponding *in vivo* studies have also shown the accumulation of ubiquitin-conjugated p53 produced by HPV E6 upon treating the cells with proteasome inhibitors (Camus, Higgins et al. 2003, Stewart, Ghosh et al. 2005, Camus, Menendez et al. 2007). Camus *et. al.* in 2003 showed that the six lysine residues present at the carboxyl terminus of p53 are not necessary for its HPV E6-mediated degradation (Camus, Higgins et al. 2003), but are known to be indispensable for ubiquitination by Mdm2 (Rodriguez, Desterro et al. 2000). Therefore, it implies that mechanistically the degradation of p53 by E6 and by Mdm2 differ.

Like the E6/E6AP-directed degradation of p53, other substrates were also found to be degraded by the E6/E6AP complex, as shown in Figure 8. These include c-myc (Gross-Mesilaty, Reinstein et al. 1998), Bak (Thomas and Banks 1998), E6TP1 (Gao, Kumar et al. 2002), and NFX1 (Gewin, Myers et al. 2004). High-risk HPV E6 proteins have also been shown to induce the degradation of a plethora of PDZ domain-containing proteins, including the human homologue of the Drosophila tumour suppressor protein discs large, hDlg (Gardiol, Kuhne et al. 1999, Pim, Thomas et al. 2000); hScrib (Nakagawa and Huibregtse 2000); the MAGI family of proteins MAGI-1, MAGI2 and

MAGI-3 (Glaunsinger, Lee et al. 2000, Thomas, Laura et al. 2002); MUPP1 (Lee, Glaunsinger et al. 2000); TIP2 (Favre-Bonvin, Reynaud et al. 2005); DLG4 (Handa, Yugawa et al. 2007); PTPN3 (Jing, Bohl et al. 2007); Cal (Jeong, Kim et al. 2007); PATJ (Storrs and Silverstein 2007); PTPN13 (Spanos, Hoover et al. 2008). Additionally, E6 also interacts with ZO-2 and β-syntrophin in a PBM dependent manner. It has been reported that the ablation of E6 expression decreases ZO-2 protein levels and biologically hampers the migration potential of E6 expressing cells (Thomas, Myers et al. 2016). High-risk HPV E6s have a Class 1 PDZ-binding motif (XS/TXV/L) at their extreme carboxy termini that is absent from the low-risk HPV E6 proteins. Through this PDZ binding motif (PBM) they bind to the PDZ domain(s) of these cellular substrates (Kiyono, Hiraiwa et al. 1997, Lee, Weiss et al. 1997, Thomas and Banks 2018). However, it remains unclear whether E6AP plays a central role in ubiquitination of these substrates, as some have been shown to be degraded in an epithelial cell line lacking E6AP (Massimi, Shai et al. 2008). Introduction of the PDZ-binding motif from high-risk into low-risk HPV E6 is sufficient to introduce the ability to induce the degradation of PDZ domain-containing substrates (Pim, Thomas et al. 2000), and it may be noted that low-risk HPV E6 proteins exhibit a low affinity for E6AP (Scheffner, Werness et al. 1990, Scheffner, Huibregtse et al. 1993) but nonetheless interact in vivo (Brimer, Lyons et al. 2007, Drews, Brimer et al. 2020). Interestingly, E6AP not only aids in E6-directed degradation of cellular substrates but also stabilizes the E6 protein levels as depicted in Figure 8. Tomaić et al in 2009, showed that knockdown of E6AP from cervical cancer-derived HeLa cells results in a dramatic decrease in levels of endogenously expressed E6 protein, and also results in a decreased E6 half-life (Tomaic, Pim et al. 2009). Therefore, all these studies suggest that another E3 ligase may exist that is capable of targeting the ubiquitination of E6 substrates as well as E6 itself as hypothesised in Figure 8.

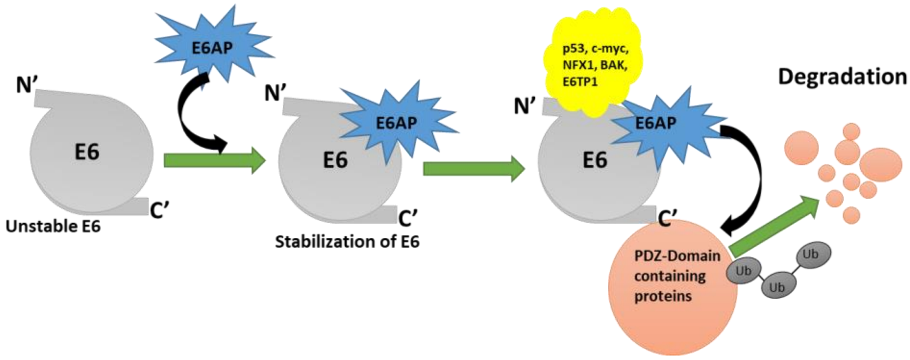
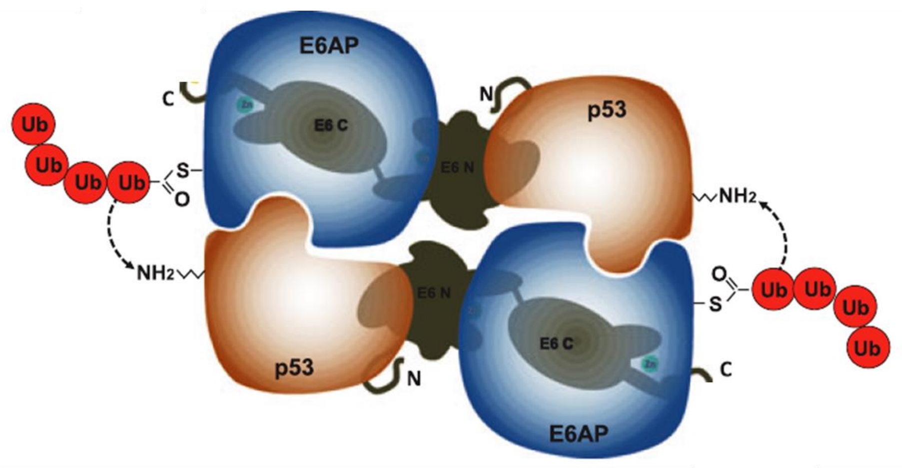


Figure 8: Schematic diagram of the E6, E6AP and target protein complex: HPV E6 oncoprotein interacts with HECT type E6AP E3 ligase. This interaction stabilizes E6 oncoproteins and also facilitates the ubiquitination of several host cell proteins as shown. This, in turn, helps the HPV to complete the viral life cycle and also can result in inducing HPV-mediated carcinogenesis.

The E6, E6AP and p53 complex

One of the most studied oncogenic activities of high-risk HPV E6 proteins is the ability to target the tumour suppressor p53 for degradation (Werness, Levine et al. 1990). This is accomplished by E6 by recruiting the E3 ubiquitin ligase E6AP (Huibregtse, Scheffner et al. 1991, Scheffner, Huibregtse et al. 1993) by directly binding to a conserved LXXLL motif positioned in a, presumably, natively unfolded region of the ligase (Huibregtse, Scheffner et al. 1993, Chen, Hong et al. 1998). This interaction alters E6AP substrate specificity via some unknown mechanism that permits the recruitment and polyubiquitination of p53, which is subsequently degraded by the 26S proteasome (Scheffner, Nuber et al. 1995). It is thought that any mutation in E6 that ablates its ability to bind LXXLL also abrogates its interaction with p53, suggesting that the LXXLL motif present in E6AP plays a very crucial role in E6-mediated degradation of p53 (Liu, Chen et al. 1999, Kao, Beaudenon et al. 2000, Cooper, Schneider et al. 2003).

Interaction of HPV-16 E6 with E6AP leads to the dimerization and self-ubiquitination of E6AP (Nuber, Schwarz et al. 1998). However, mutations made on the dimerization surface of HPV-16 E6 lead to the disruption of both E6 dimerization and p53 degradation *in vitro;* thus, implying that the dimerization of E6 is functionally linked with the p53 degradation (Zanier, ould M'hamed ould Sidi et al. 2012). It has been found that when an E6, mutated in its dimerization domain, was transiently expressed in HeLa cells, it induced senescence, presumably due to the dominant-negative interaction with both E6AP and p53 (Ristriani, Fournane et al. 2009). All these studies explain the absolute requirement for E6's ability to multimerize, which occurs via self-association with its N-terminal domain as depicted in Figure 9 (Pim, Bergant et al. 2012, Zanier, ould M'hamed ould Sidi et al. 2012), in order to initiate the transfer of ubiquitin molecules from E6AP to p53 that eventually leads to p53 degradation (Scheffner, Huibregtse et al. 1993).



(Modified from Pim, Bergant et al. 2012)

Figure 9: Model representing E6/E6AP/p53 trinary complex: E6 dimerization mediated by E6N, bringing together E6AP and p53 on the opposite sides of the dimer, thereby facilitating the ubiquitination and degradation of p53.

Recently, a study by Sailer et al, (2018) showed that E6, when it binds to E6AP induces a conformational change in the structure of E6AP. As a consequence, E6 and p53 are positioned in the direct vicinity of E6AP’s catalytic center, wherein E6 functions as an adaptor protein facilitating the transfer of ubiquitin moieties from E6AP to p53, that leads to the degradation of p53 (Sailer, Offensperger et al. 2018).

Apart from p53 degradation, E6 is also known to modulate the transcriptional regulatory activities of p53. In a normal scenario, when the cells are under stress in response to DNA damage, ATR is activated to phosphorylate p53 and also to block its MDM2-directed degradation, thereby allowing it to accumulate. However, in HPV-positive cervical cancer cells, E6 blocks this transactivation of p53 by delaying ATR activation (Wallace, Robinson et al. 2012, White, Walther et al. 2014). This occurs through p300/CBP, which normally acetylates p53 and acts a co-activator, upregulating p53 transactivation, as well as downstream effects of growth arrest and/or apoptosis (Grossman 2001). HR HPV E6 binds to p300/CBP at different sites and prevents the transactivation of p53 by inhibiting the activation of pro-apoptotic NF-KB (Patel, Huang et al. 1999, Zimmermann, Degenkolbe et al. 1999). E6 also targets and degrades hADA3 and TIP60, other acetyl transferases acting as p53 transcriptional co-activators (Kumar, Zhao et al. 2002, Jha, Vande Pol et al. 2010) . In addition, E6 masks the nuclear localization signal of p53 and sequesters it into the cytoplasm, thereby preventing it from carrying out its transcriptional activity in the nucleus (Mantovani and Banks 2001). To summarize, E6 degrades p53 and in part also prevents its activation through several additional mechanisms, indicating its importance in the viral life cycle as well as in cancer progression. This implies the existence of scenarios where p53 is degraded directly by the E6-E6AP complex, but others where p53 is still present but functionally compromised by E6 through these other mechanisms. What controls these different selective functions of E6 remains to be determined.

Telomerase activation, immortalization and transformation.

Another critical function of HPV E6 that can contribute to oncogenesis is the activation or upregulation of telomerase, an enzyme responsible for adding the repetitive sequence to the end of the chromosomes, thus stabilizing the length of the telomere, which is crucial for avoiding cellular senescence (Klingelhutz, Foster et al. 1996). E6-directed increase in the activity of hTERT occurs independently of p53 degradation (Klingelhutz, Foster et al. 1996, Kiyono, Foster et al. 1998). It is thought to be carried out through a complex series of transactivation events, including its interaction with c-Myc and Sp1 upregulating the E-box-dependent transcription of hTERT for immortalization (Oh, Kyo et al. 2001, Veldman, Liu et al. 2003). Another study model also proposes that E6 cooperates with NFX-123, in stabilizing the hTERT transcripts (Katzenellenbogen, Vliet-Gregg et al. 2009). On the other hand, NFX1-91 (a transcriptional repressor of TERT) is targeted for polyubiquitination by E6/E6AP, thereby removing it from the hTERT promoter, ultimately leading to enhanced transcriptional activation of hTERT (Gewin, Myers et al. 2004, Xu, Katzenellenbogen et al. 2013). The reason why HPV activates telomerase is not clearly known. One of the possible reasons could be that telomerase activation increases the life-span of the keratinocyte and also induces the DNA damage response, providing an advantage to the virus for its own replication (Vande Pol and Klingelhutz 2013, Wallace and Galloway 2015).

Attenuation of apoptotic signaling

High-risk HPV E6 targets a number of pro-apoptotic proteins, other than p53, controlling both intrinsic and extrinsic apoptotic signalling cascades. The intrinsic apoptotic signalling pathways are triggered in response to the internal signals arising from numerous sources, including lack of growth factors and DNA damage. Once activated, all the signals coalesce at mitochondria, resulting in release of cytochrome C into the cytoplasm, eventually leading to cell death. E6 increases the abundance of anti-apoptotic proteins like Bcl2, Survivin and c-IAP2 (Du, Chen et al. 2004, Yuan, Fu et al. 2005, Borbely, Murvai et al. 2006, Wallace and Galloway 2015) and degrades the key pro-apoptotic protein, Bak (Thomas and Banks 1998, Thomas and Banks 1999), thereby disrupting the balance between anti-apoptotic and pro-apoptotic signalling molecules, involved in the regulation of this pathway.

HPV E6 also blocks the activation of the extrinsic apoptotic pathway, in response to the signals coming from outside the cells at multiple regulatory points via binding to the tumour necrosis factor receptor 1 (TNF R1) (Filippova, Song et al. 2002), the adaptor molecule Fas-associated death domain (FADD) (Filippova, Parkhurst et al. 2004), and procaspase 8 (Filippova, Johnson et al. 2007). E6 binds to the death effector domains (DEDs) of FADD and procaspase 8, which, in turn, mediates the accelerated degradation of both. Thus, the interaction of E6 with these proteins make them incapable of efficiently participating in transmitting the apoptotic signals, thus giving rise to the protection against apoptosis-inducing stimuli (Tungteakkhun and Duerksen-Hughes 2008).

Cell polarity, adhesion and proliferation control

Epithelial tissues possess a very typical and well-characterized polarised cellular architecture with specialised cell-cell junctions, that include desmosomes, tight junctions and adherens junctions. Cell polarization allows these cells to sense and to elicit the proper spatio-temporal responses to signals that arise from the surrounding microenvironment. A number of signalling and polarity complexes are involved in maintaining the cell junctions and in the establishment of cell polarity (Halaoui and McCaffrey 2015). In general, cell polarity is maintained by the coordinated interplay between a conserved family of proteins that typically form three major polarity control complexes: the Crumbs, Par and Scribble complexes. Each of these complexes are involved in controlling and defining the polarity of different regions of the cell. The Crumbs complex, consisting of Crumbs3, Pals1 and PatJ, controls apical polarity (Suzuki and Ohno 2006, Laprise 2011, Pocha and Knust 2013); the Par complex is a dynamic complex, which mainly comprises the Par2, Cdc42, Par6 and aPKC (atypical protein kinase C) proteins, and is also known to interact with the Crumbs complex (Suzuki and Ohno 2006); while the Scribble complex, maintains the basolateral polarity of the cell and mainly consists of scaffolding proteins Scribble, Dlg and huLgl1 (Pim, Bergant et al. 2012). Thus, it is important to remember that any kind of alteration or disruption in the cell polarity regulators, make them unresponsive to growth inhibitory signals, and could eventually circumvent differentiation, senescence or apoptosis. One of the prime characteristics of high-risk HPV E6 oncoproteins in mediating tumorigenesis has been linked to their ability to target these cellular proteins that mediate cell–cell adhesion, polarity, and control proliferation (McCaffrey and Macara 2011, Thomas and Banks 2018).

All high-risk HPV E6 oncoproteins possess a short carboxy terminal stretch of amino acids that confers interaction with cellular proteins that possess a PDZ domain (PSD-95/DLG/ZO-1). This so-called PDZ binding motif (PBM) is absent from the non-cancer-causing HPV E6 oncoproteins, and is thought to be a major element in the ability of the high-risk viruses to cause cancer (Songyang, Fanning et al. 1997). The E6 proteins from high-risk HPV types possess a canonical X-S/T-X-V/L_COOH consensus site, with a significant degree of variations within this site in different HPV E6 types. Through this PBM, E6 has been shown to recognize (albeit with differing affinities) many different PDZ domain- containing cellular proteins, including the major components of the cell polarity control machinery; the Crumbs complex (Latorre, Roh et al. 2005, Storrs and Silverstein 2007), the Par-aPKC (Facciuto, Bugnon Valdano et al. 2014) and the Scribble-Dlg complexes (Gardiol, Kuhne et al. 1999, Nakagawa and Huibregtse 2000, Thomas, Massimi et al. 2005). Furthermore, HPV E6 oncoprotein also targets the MAGI family of proteins (Glaunsinger, Lee et al. 2000, Thomas, Laura et al. 2002), that are known to play a crucial role in maintaining junctional integrity and stability.

Numerous studies have reported that Scribble and Dlg are *bona fide* binding partners of high-risk HPV E6 oncoproteins, which further leads to their degradation by E6, both *in vitro* as well as *in vivo* (Gardiol, Kuhne et al. 1999, Nakagawa and Huibregtse 2000, Kranjec and Banks 2011). It has also been shown that the ability of HPV E6- and E7-induced transformation in rodent epithelial cells is inhibited by the overexpression of Scribble, suggesting its potential role as tumour suppressor (Nakagawa and Huibregtse 2000, Nguyen, Nguyen et al. 2003, Thomas, Massimi et al. 2005). Indeed, deregulation and mislocalisation of Scribble in a murine model of breast cancer has potent oncogenic activity affecting subcellular localization of PTEN and activating an mTOR signalling pathway (Zhan, Rosenberg et al. 2008, Feigin, Akshinthala et al. 2014). In the case of cervical cancer, the expression patterns of Scribble and Dlg are severely perturbed during tumour progression (Watson, Rollason et al. 2002, Cavatorta, Fumero et al. 2004, Lin, Steller et al. 2004). For instance, in normal tissue, Dlg is localised at regions of cell-cell contact, but in cervical intraepithelial precursor lesions, its localisation is altered and has a cytoplasmic distribution, while a loss of Dlg is observed in late stages of invasive cervical cancer (Cavatorta, Fumero et al. 2004). A similar trend is observed in the case of Scribble, which is re-localized from cell-cell contacts in normal squamous cells to the cytoplasm in early lesions, followed by a gradual reduction in its expression levels as the tumour develops (Nakagawa, Yano et al. 2004). It is important to remember that E6 does not mediate the degradation of the entire pool of Scribble and Dlg, but instead targets only a subset of the proteins. HPV 18E6 has been shown to target only the phosphorylated and nuclear fraction of Dlg for proteasomal degradation. Ironically, in certain contexts, both Dlg and Scribble show pro-oncogenic activities, which enhance tumour aggressiveness. For example, a study by Krishna Subbaiah *et al* in 2012, showed that stimulation of high levels of RhoG activity by E6 depends upon its interaction with Dlg and the RhoG guanine nucleotide exchange factor SGEF, resulting in increased tumour invasion potential (Krishna Subbaiah, Massimi et al. 2012). Therefore, it is interesting to speculate that the redistribution or overexpression of Dlg or Scrib may induce a shift from tumour suppressor to a pro-oncogene, possibly related to changes in the pool of their binding partners, thus altering their functions, especially in the case of intermediate grade tumours, thus increasing their potential for progression to invasive cancers (Facciuto, Cavatorta et al. 2012, Ganti, Broniarczyk et al. 2015).

Apart from Dlg and Scribble, high-risk HPV E6 oncoproteins have been shown to strongly bind and target the MAGI group of proteins for degradation (Glaunsinger, Lee et al. 2000, Thomas, Glaunsinger et al. 2001, Thomas, Laura et al. 2002, Zhang, Dasgupta et al. 2007, Charbonnier, Nomine et al. 2011). Like Dlg and Scribble, only membrane and nuclear pools of MAGI-1 are susceptible to proteasome-directed degradation by both HPV-16 E6 and HPV-18 E6. As a result of this interaction, HeLa (positive for HPV 18E6) and Caski (positive for HPV 16E6) cells have been observed to lose their junctional integrity, which is rescued by re-emergence of MAGI-1 protein levels upon E6 ablation (Kranjec and Banks 2011). Also, the reintroduction of a MAGI-1 mutant that is resistant to E6-induced degradation in HPV-positive cells leads to the accumulation of ZO-1 and Par3 at regions of cell-cell contact, as well as to a marked reduction in cell proliferation and an increase in apoptosis (Kranjec, Massimi et al. 2014). These findings unravel the pathological significance of the loss of MAGI-1 in HPV-positive cervical cancer cells, which includes loss of tight junction integrity, an increase in cell proliferation and a suppression of apoptosis, all of which can be expected to boost the progression of hyperplastic lesions into metastatic cancer (Ganti, Broniarczyk et al. 2015). In addition to MAGI-1, the related proteins MAGI-2 and MAGI-3 are also targeted by high-risk HPV E6 for degradation (Thomas, Laura et al. 2002), although the biological consequences of this remain unknown.

Recent studies have highlighted a novel function of the high-risk HPV E6 PBM: regulating endocytic transport pathways, via sorting nexin 27 (SNX27). This interaction directly increases rates of cargo recycling and, as a result of this, HPV-transformed cells show high levels of glucose uptake (Ganti, Massimi et al. 2016). This E6-PDZ interaction is exceptional, in that it does not result in degradation of the PDZ domain-containing target protein, but rather modulates its function. In addition to SNX27, Thomas *et al* in 2016, have reported that high-risk HPV E6 oncoprotein interacts and stabilizes the tight junction protein ZO-2 in a PBM dependent manner and ablation in ZO-2 protein levels abrogates the wound healing process, even in the presence of the E6 and E7 oncoproteins. This suggests that some of the migration-promoting potential of E6 may be mediated through ZO-2 (Thomas, Myers et al. 2016). Additionally, the E6-PDZ domain interaction plays a significant role in maintaining the viral life cycle, as loss of this motif from the whole viral genome results in a significantly reduced replicative capacity, and ultimately in loss of episomes (Lee and Laimins 2004, Delury, Marsh et al. 2013).

Phosphoregulation of HPV E6 PBM

As described above, the E6 oncoprotein targets various PDZ domain-containing cellular proteins and modulates their functions, both during the viral life cycle and during cancer progression; suggesting that the interactions are multifunctional in nature. Intriguingly, the E6 PBM is equally multifunctional, owing to the phospho-acceptor site embedded within its PBM. This post-translational modification plays a crucial role in regulating E6 function. It was first shown that the threonine residue present within the PBM [at 156 residue of HPV 18E6], could very efficiently be phosphorylated by protein kinase A (PKA). This phosphorylation, in turn, abrogates the ability of E6 to bind its PDZ substrate Dlg (Kuhne, Gardiol et al. 2000). The molecular mechanism for this is well studied and has been reported for many PBM-PDZ ligand combinations. It has been demonstrated in previous studies that the presence of a phosphate moiety prevents the E6 PBM fitting into the limited space of the PDZ binding pocket (Ivarsson 2012). Central to the consensus sequence of the PBM is a S/T present at the -2 position in all the high-risk HPV E6 oncoproteins, as shown in Figure 10. This can often be phosphorylated either by PKA, AKT or Chk-1 (Delury, Marsh et al. 2013, Thatte, Massimi et al. 2018) kinases, however, this regulation may vary for different HPV types and may not be controlled by the same kinase in all cases. For example, HPV-16 E6 phosphorylation can be mediated by both PKA or AKT, but HPV 18E6 can only be phosphorylated by PKA (Boon and Banks 2013).

Interestingly, previous studies have shown that phosphorylation of the HPV E6 PBM inhibits its interaction with PDZ domain-containing proteins, but simultaneously confers a strong direct interaction with 14-3-3 family of proteins as shown in Figure 10 (Boon and Banks 2013). 14-3-3 proteins are a group of highly conserved acidic proteins that are abundantly present in the cell. These proteins are known to bind and regulate the functions of a large repertoire of cellular proteins involved in several processes, including apoptosis, signal transduction, tumour suppression, metabolic control and transcription; in a phospho-dependent manner (Dougherty and Morrison 2004, Jin, Smith et al. 2004, Pozuelo Rubio, Geraghty et al. 2004). Most importantly, the interaction with 14-3-3 appears to be another mechanism by which E6 can inhibit the activity of p53. Previous studies had shown that the loss of viral episomes which is observed following mutation of the E6 PBM could in fact be rescued by mutation of p53, indicating a direct link between and intact PBM and p53 inactivation (Brimer and Vande Pol 2014). One link would appear to be 14-3-3, which is a known regulator of p53 nuclear expression, However phospho-E6 interacts with 14-3-3 and inhibits p53 transcriptional activity (Thatte, Massimi et al. 2018). It therefore seems likely that the E6 PBM function will be differentially regulated through the progression of the viral life cycle, in the context of both recognising different PDZ-containing substrates and interacting with phosphorylation-dependent cellular proteins, such as 14-3-3.

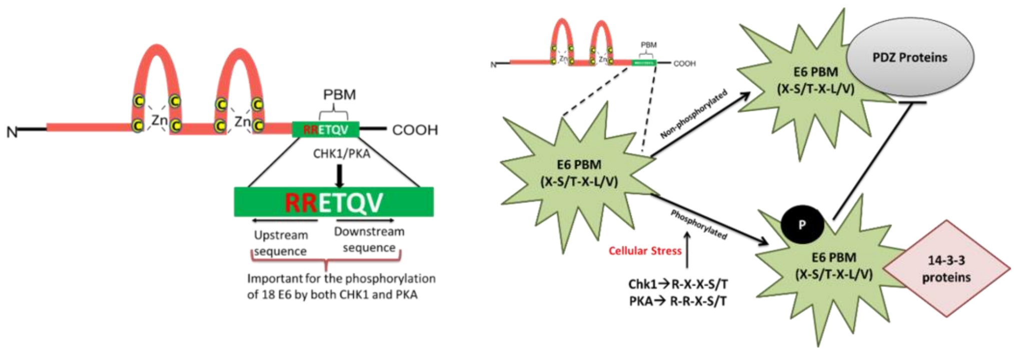


Figure 10. Schematic diagram of E6 PBM: A representative picture of high-risk HPV types that shows the presence of the conserved PBM sequence (x-T/S-x-V/L) at the C-terminus end (shown in green in Left panel). This motif is absent in low-risk types. PBM when phosphorylated abrogates binding of E6 with PDZ proteins and confers binding with 14-3-3 proteins (shown in right panel).

HPV Manipulation of the DNA Damage Response pathways (DDR)

Over the past several millions of years, HPVs have co-evolved with their host cells and have adapted several cellular mechanisms to ensure the completion of the viral life cycle. This is known to be achieved, at least in part, by HPV exploiting the host cell DDR pathway to benefit itself by promoting its own replication at different stages of the viral life cycle. Use of the repair machinery to replicate viral DNA would be advantageous since it would allow the virus to synthesize its DNA without competing with that of the host cell (Chow, Duffy et al. 2009). Several reports have demonstrated that HPV induces the activation of both ATM and ATR pathways, as well as the FA (Fanconi Anæmia) pathway, independently of any external DNA damage stimuli. Therefore, studies based on understanding the relationship between HPV and DNA damage may provide us with useful insights into the control of HPV-associated cancers.

Regulation of the ATM pathway

Studies have shown that cells infected with HPV constitutively express activated ATM, which is apparently crucial for the productive life cycle of all high-risk HPVs. Upon infection by HPV-31, as well as by HPV-16 and HPV-18, E7 initiates the activation of the ATM pathway, which is maintained throughout the viral life cycle. Several ATM substrates, such as CHK2, NBS1 and BRCA1 are phosphorylated in HPV-infected cells, with the levels of phosphorylation remaining high through the differentiation process (Moody and Laimins 2009, Sakakibara, Mitra et al. 2011). In addition to E7, it has been shown that the higher-expression levels of E1 protein can also elicit the activation of ATM pathways, most likely by initiating the replication from the pseudoviral origin situated in the cellular DNAs that subsequently leads to stalled replication forks (Kadaja, Isok-Paas et al. 2009, Moody and Laimins 2009, Sakakibara, Mitra et al. 2011).

It has been reported that, at the site of HPV replicating genomes in HPV-positive cells, the components of the ATM pathway form discrete puncta in the nucleus, together with downstream cellular factors that are involved in carrying out homologous recombination, such as Rad51, Brca1 and pRPA S33. This therefore raises the possibility that homologous recombination is involved in amplification of the HPV genome in undifferentiated as well as in differentiated epithelium (Gillespie, Mehta et al. 2012) . Moody *et al* in 2009 showed that inhibiting the activity of ATM by treating

HPV-positive cells with kinase inhibitor adversely affects the differentiation-dependent viral genome amplification, but showed no effect on episomal maintenance in undifferentiated cells (Moody and Laimins 2009). This work opened up similar lines of investigation in many other DNA tumour viruses, where similar mechanisms of activation of the DDR to complete viral DNA replication have been reported. Phosphorylated STAT-5 have been shown to activate the ATM DNA damage pathway and promotes the amplification of the viral genome in differentiated keratinocytes (Hong and Laimins 2013). Additionally, the ATM pathway also phosphorylates SMC1, which remains constitutively active in both differentiated and undifferentiated cells and aids in the amplification of the HPV genome. SMC1 is a cohesion protein, involved in proper chromosomal segregation during mitosis. Phosphorylated SMC1 localizes to foci containing complexes of γ-H2AX and pCHK2 in the nucleus, where it directly binds to HPV DNA and forms a complex with the CTCF insulator, present in the L2 late region. Inhibiting the binding of pSMC1 with CTCF quickly destroys the capability of the virus to be stably maintained as an episome or to amplify upon differentiation (Mehta, Gunasekharan et al. 2015). Recruitment of CTCF to HPV genome has also been shown to regulate the expression and transcript processing of the viral early gene region (Paris, Pentland et al. 2015). Taken together, these findings suggest that there is a critical requirement for ATM activation during the viral life cycle.

Regulation of ATR pathway

Little is known about the role of activation of ATR pathways in the context of the HPV life cycle. However, it has been shown that during the productive viral life cycle, rapid viral replication load may lead to replicative stress in the HPV-infected cell, which subsequently activates the ATR pathway. This replicative stress-mediated DDR leads to the accumulation of ATR pathway proteins at HPV-18 replication foci (Kadaja, Isok-Paas et al. 2009, Gillespie, Mehta et al. 2012, Reinson, Toots et al. 2013). Also, it has been reported that as the HPV-positive cells differentiate, the levels of RPA32 protein (known to protect exposed ssDNA during the replication process) increase at replication foci, suggesting a response to increased viral replication (Gillespie, Mehta et al. 2012). Subsequent studies have shown that cells containing HPV genomes, or expressing E6/E7 proteins alone, have increased levels of ATR and its downstream target, CHK1. It has been reported that STAT-5 mediated the activation of ATR in differentiating HPV-positive cells by directly regulating the transcription of TopBP1 protein, an upstream activator of ATR pathway (Hong, Cheng et al. 2015).Targeting ATR, by using inhibitors specific for either pATR or pCHK1, reduces the stability of the viral episomes in undifferentiated as well as differentiated HPV-positive epithelium (Edwards, Helmus et al. 2013, Hong, Cheng et al. 2015). It has been recently shown that the activated ATR in HPV-positive cells also increases phosphorylation of p62 which results in decreased levels of GATA 4, which in turn controls the expression of genes associated with the inflammatory response (Hong, Li et al. 2020). Thus, these studies suggest that the ATR pathway may not only play a crucial role in HPV episomal maintenance but may also the target inflammatory response in order to allow persistent HPV infection (Hong, Li et al. 2020).

Regulation of the FA pathway

Studies have shown that the high-risk, but not low-risk, HPV E7 oncoprotein controls the FA pathway by upregulating the transcription of several genes belonging to this pathway. This occurs through an Rb-dependent mechanism (Hoskins, Gunawardena et al. 2008). Activation of the FA pathway by E7 elicits the recruitment of FANCD2 and BRCA2 to chromatin (Spardy, Duensing et al. 2007). It has also been shown, in the case of HPV-31-positive cells, that FANCD2 localizes to the viral replication foci and also binds directly to many sites along the viral genome. This binding takes place in both differentiated and undifferentiated cells, but gradually decreases as the cells differentiate. In addition to this, depletion of FANC2 from these cells led to reduced maintenance of viral episomes, which might eventually increase the probability of viral genome integration into the host cell genome (Spriggs and Laimins 2017).

Aim of the thesis

The high-risk Human Papillomavirus (HPV) E6 oncoprotein is known to contribute to human malignancy by targeting several of its cellular substrates through the ubiquitin-mediated degradation pathway. Previous studies have revealed that E6 interacts with the E6AP ubiquitin-protein ligase to stabilize itself (Tomaic, Pim et al. 2009), and also directs E6AP's ubiquitylation activity toward several specific cellular proteins, of which the most important are p53 and the PDZ domain-containing proteins. However, the role of E6AP in the degradation of many other E6 substrates is still ambiguous, as studies have shown that E6 is able to induce the degradation of certain target substrates in the absence of E6AP (Massimi, Shai et al. 2008). However, all this is complicated by the fact that loss of E6AP also induces a reduction in E6 stability.

- So, the first objective of my PhD work focuses on understanding the interaction of the E6 oncoprotein with the ubiquitin proteasome pathway. The aim here is to determine which ligase is involved in E6 degradation when it is not bound to E6AP, and also which cellular substrates can be degraded by E6 in E6AP-independent manner.

Cancer-causing HPV E6 oncoproteins contain a well-characterised phospho-acceptor site within the PDZ (PSD-95/Dlg/ZO-1) binding motif (PBM) at the C-terminus of the protein. Previous studies have shown that the threonine or serine residue in the E6 PBM is subject to phosphorylation by several stress-responsive cellular kinases, upon the induction of DNA damage in cervical cancer-derived cells (Thatte, Massimi et al. 2018). However, there is little information about the regulation of E6 phosphorylation in the absence of DNA damage and whether there may be other pathways by which E6 is phosphorylated.

- So, the second objective of my PhD work focuses on understanding the role of phospho-regulation of HPV E6 oncoprotein without inducing DNA damage exogenously in the cervical cancer-derived cell line.

Material and Methods

Cell culture

HeLa, SiHa, H1299 cells and HEK293 E6AP knockout cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, #31885-023) supplemented with 10% fetal bovine serum (GIBCO, #10270-106), penicillin-streptomycin (100 U ml^{-1}), and glutamine (300 µg ml^{-1}) (GIBCO, 10378-016) at 37°C in a humidified air incubator containing 10% CO_2.

NIKS were grown in Ham’s nutrient mixture F-12 medium (GIBCO, #21765-029) supplemented with 5% fetal bovine serum (GIBCO, #10270-106), 0.4µg/ml hydrocortisone, 5 µg/ml insulin, 10ng/ml EGF, 24µg/ml adenine and penicillin-streptomycin (100 U ml^{-1}) at 37°C in a humidified air incubator containing 10% CO_2.

Chemicals, Inhibitors and antibodies

The inhibitors used for the experiments in this study were as follows: 100 nM UCN01 CHK1 inhibitor (Sigma Aldrich, #U6508), 10 µM H-89 PKA inhibitor (Sigma Aldrich, #B1427) and 30 µM DNA PK inhibitor (Abcam, NU7026). DNA damaging agent Teniposide (Sigma Aldrich, #SML0609) was used at a concentration of 5 µM, 30µM p53 transcriptional activity inhibitor α-pifithrin (Merck, #p4359). The proteasome inhibitor used was CBZ (MG132 Z-Leu-Leu-Leu-al, Sigma Aldrich # C2211).

Primary antibodies used were the rabbit polyclonal HPV-18 E6 phospho-specific antibody (1:500, custom-made by Eurogentec) was generated using H2N-RQERLQRRRET(PO3H2)QV-COOH peptide in rabbits and subjected to affinity purification, Mouse monoclonal 18E6 (1:500, Santa Cruz, #sc365089) and Mouse monoclonal p53 (1:2000, Santa Cruz, #sc126), Mouse monoclonal E6AP (1:500, BD Biosciences, #611416), Mouse monoclonal α-tubulin (1:20,000, Cell Signaling Technology, #T5168), Mouse monoclonal anti-FBXO4 (1:500, Santa Cruz, #sc376872), Mouse monoclonal anti-Flag (1:1000, Sigma Aldrich, #F3165), Mouse monoclonal anti-myc antibody (1:1000, Santa Cruz, #sc-40), Rabbit polyclonal anti-GFP (1:2000, Abcam, #ab6556) Rabbit monoclonal $^{pS139}\gamma$H2AX (1:1000, Cell Signaling Technology, #20E3), Mouse monoclonal anti-18E7 (1:500, Santa Cruz, #sc365035), Mouse monoclonal anti-16E7 (1:500, Santa Cruz, #sc65711), Mouse monoclonal actinin (1:4000, Santa Cruz, #sc17829), Mouse monoclonal β-Gal (1:4000, Promega, #Z378B), Mouse monoclonal HA-tag-Peroxidase (1:4000, Sigma Aldrich, #H6533-1VL), Goat polyclonal anti-HA (1:500, Abcam #ab5069), Alexa Fluor 546-

conjugated anti-goat (1:500, Invitrogen, #A11056) and Alexa Fluor 647-conjugated anti-mouse (1:500, Life Technologies, #A31571), followed by HRP conjugated anti-rabbit (1:2000, DAKO, #P0260) and anti-mouse secondary antibody (1:2000, DAKO, #P0217).

Plasmids and cloning

The plasmids used were as follows: pGWI HA-Scribble (Thomas, Massimi et al. 2005), pCDNA3 V5-MAGI-3 (Thomas, Massimi et al. 2005), pCDNA3 FLAG-E6AP CA and pGWI myc-E6AP WT (Tomaic, Pim et al. 2009), pGWI HA-DLG1 (Thomas, Massimi et al. 2005) and pCDNA His-LacZ (Invitrogen). p21-Luciferase, MDM2-Luciferase, and Renilla luciferase, FLAG-p53, pGWI-18 E6, and pGWI-18 E6 ΔPBM have been described previously (Thatte, Massimi et al. 2018). The GST, HPV-11 E6 GST, HPV-16 E6 GST, HPV-18 E6 GST, HPV-31 E6 GST, HPV-33 E6 GST, HPV-51 E6 GST, HPV-18 E6 T156E GST, HPV-58 E6 GST and HPV 18 E6_ S82A_T156E GST, HPV-16 E7-GST, HPV-16 E7 N'-GST, HPV-16 E7 C'-GST and HPV-18 E7 GST -fusion proteins have been described previously (Tomaic, Pim et al. 2011, Boon and Banks 2013, Boon, Tomaic et al. 2015, Szalmas, Tomaic et al. 2017). pcDNA3 Flag-tagged FBXO4 was a kind gift from Prof. J. Alan Diehl from the University of Pennsylvania, USA, HA-tagged UB has been described previously (Thatte and Banks 2017). The C-terminal FLAG/HA-tagged-16E7 and 18E7 were kind gifts from Karl Munger, Harvard Medical School, USA (Gonzalez, Stremlau et al. 2001).

pHAHA-Empty GFP and pHAHA-GFP18E6 were made by sub-cloning GFP from peGFP C1 plasmid (Addgene, # #6084-1) using Nhe1 and BamH1 restriction sites into pHAHA empty vector (Addegene, #12617) (Luu, Zhou et al. 2005). We then used pHAHA-Empty GFP to clone 18E6. To make pHAHA-GFP18E6, we first amplified 18E6 from pEGFP-18E6 plasmids, using EcoR1 and Sal1 restriction sites and then cloned it into the pHAHA-GFP vector. The primer sequence used are listed below:

GFP18E6_F: ATA GAA TTC ATG GCG CGC TTT GAG GAT C and

GFP18E6_R: CCT GTC GAC TTA TAC TTG TGT TTC TCT GC

The 18E6 ET*PBM mutant was generated by using the Gene Art Site-Directed mutagenesis kit (Invitrogen) and the primer sequences are as follows: 18E6GST ET*_F: CGACGCAGAGAAACATGATATTAAGTATGCATGG, 18E6GST ET*_R:

CCATGCATACTTAATATCATGTTTCTCTGCGTCG. GST fusion HPV-35 E6, HPV-39 E6, HPV-56 E6 and HPV-68 E6 proteins (the kind gift of Carina Eklund, Karolinska Institute) were generated by sub-cloning them from their respective plasmids using BamH1 and EcoR1 restriction sites. The primer sequences used for the same are listed as follows:

35 E6 F: GGTGGATCCATGTTTCAGGACCCAGCTG and

35 E6_R: GGGGAATTCTTACACCTCGGTTTCTCTACG,

39 E6_F: GTTGGATCCATGGCGCGATTTCACAATC and

39 E6_R: GGGGAATTCTTATACTTGGGTTTCTCTTCG,

56 E6_F: GGCGGATCCATGGAGCCACAATTCAAC and

56 E6_R: GGCGAATTCTTATACTGTAGATTCTCTAG,

68 E6_F: GCTGGATCCATGGCGCTATTTCACAAC and

68 E6_R: GGGGAATTCTTAAACTTGTGTTTCTTGACG

Transient Transfection

Cells were seeded in an appropriate dish with 50–60% confluency. After 24 h, media was changed and solution A, containing the respective DNA in TE and $CaCl_2$ was prepared. After that, solution B i.e. 2X HBS was added dropwise to the solution A along with gentle mixing followed by incubation for 30 minutes at room temperature. The transfection mixture was added dropwise to the desired plate. Cells were then harvested for further analysis.

Solution A: Required amount of DNA diluted in 100 µl of TE buffer + 11.2 µl of 2.5M $CaCl_2$

Solution B: 100 µl of 2X HBS, pH- 7.12 (50mM HEPES pH-7, 280mM NaCl, 1.5mM $Na_2HPO_4.7H_2O$)

Immunoblotting

Cells were harvested and lysed in Laemmli sample lysis buffer (Composition: 4% SDS; 20% glycerol; 0.004% bromophenol blue; 0.125M Tris-Cl, pH 6.8, 10% 2-mercaptoethanol). The whole cell extracts were then were then electrophoresed in SDS-polyacrylamide gels, transferred to Nitro-cellulose Membrane (0.22µm, Amersham #10600001). The membrane was then probed for different proteins using the relevant primary antibodies, followed by the appropriate HRP-conjugated secondary antibody. Detection of peroxidase activity was performed using the western lightening ECL reagent (Amersham, #RPN2106 and GE healthcare, #17174088).

Generation of GFP-tagged 18E6 and empty-GFP stable cell lines in HEK293 E6AP K/O cells

HEK293 E6AP K/O cells were transfected with plasmids expressing pHAHA-GFP empty and pHAHA-GFP18E6 containing a hygromycin-resistance gene (Thermo Fischer # 10687010) using the $CaCl_2$ transfection protocol. After 24 hours, cells were selected using hygromycin at a concentration of 200μg/ml for 2 weeks, changing the media containing hygromycin antibiotic every 2 days. After 2 weeks, 10 cells per 10cm petri-dish were seeded in multiple plates, that were then allowed to form colonies for the next 2 weeks. Around 20 colonies were picked for each pHAHA-Empty GFP and pHAHA-GFP18E6, followed by validation of the protein expression using western blotting.

siRNA Library Screening

The human cellular ubiquitin ligases siRNA library was purchased from Dharmacon containing ~598 target genes (siGENOME SMARTpools of 4 siRNAs against each gene). To perform the screening, siRNAs were transferred robotically from stock library plates to poly-L lysine-coated 384-well plates (Perkin Elmer), leaving 2 columns empty for the addition of all relevant controls, which includes buffer, non-targeting siRNAs, siRNA against UBC gene, and proteasome inhibitor CBZ. siRNAs were reverse-transfected into HEK293 E6AP GFP18E6 stable cells at a final concentration of 50nM. After 72 hours, PFA was added to each well without removing the media from the wells from the stock of 16% to give a final concentration of 4% for 15 min at room temperature. Cells were then washed twice with PBS and permeabilized using 0.5% Triton-X in PBS for 20 min at room temperature. After this, cells were washed thrice with PBS and nuclei were stained using Hoechst 33342. Images were acquired using ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices) at a magnification of 10; a total of 9 images were acquired for each well and replicate.

Image analysis was performed using the ‘Multi-Wavelength Cell Scoring’ application module implemented in MetaXpress software (Molecular Devices). Nuclei were segmented based on Hoechst 33342 staining, and cells were then classified as positive or negative, depending on the total area of GFP18E6 staining. The screening was performed at the ICGEB High-Throughput Screening Facility (http://www.icgeb.org/high-throughput-screening.html).

XTT- Cell Viability assay

Cells were reverse transfected with the relevant siRNAs in a 96-well plate in a final volume of 100μl culture medium per well. After 72 hours' incubation, 50μl of the XTT labelling mixture (prepared by mixing 5ml of XTT labelling reagent with 0.1ml electron coupling reagent) (Roche, #11465015001) was added to each well, followed by incubation for 5 hours in a humidified chamber with 10% CO_2 maintained at 37°C. After this incubation period, the formazan dye formed was quantitated using ELISA reader at a wavelength of 490. The measured absorbance directly correlates to the number of viable cells.

Fluorescence activated cell sorting (FACS)

First cells were reverse-transfected with different siRNAs in 6-well plate. After 72 hours' incubation, cells were washed with PBS, trypsinized and centrifuged at 1500 rpm for 5 min. After this, cells were re-suspended in 1ml PBS, out of which 200µl was taken to perform FACS analysis and the rest was used to perform western blotting. The 200µl cell suspension in PBS was centrifuged at 1500 rpm for 5 min and the pelleted cells were re-suspended in 500µl of incubation buffer (composition: 10mM HEPES, pH7.4, 140mM NaCl, 5mM $CaCl_2$) containing Annexin-V Fluos (10µl per 500µl incubation buffer) (Roche, # 11828681001) and Propidium iodide (PI) (10µl from stock of 50µg/ml per 500µl incubation buffer) (Sigma Aldrich, #P4864) followed by incubation for 15 min at room temperature. The numbers of live and apoptotic cells under each condition were detected using FACS (BD FACS Celesta). Analysis of data was performed using FlowJo software.

siRNA Transfection

Cells were seeded at a confluence of 30-40% in a 6-well plate and after 24h, they were transfected with siRNAs against E6AP (Dharmacon, SMARTPool #L-005137-00-0005), E6/E7 (Dharmacon, #HR1ZN-000327), p53 (Dharmacon, #J-003329-14-0005), FBXO4 (Dharmacon, #J-012433-05-0005), Luciferase (Dharmacon, #D-002050-0-1-

20), Scramble (Dharmacon, #D-001700-01-20), 18E6/E7 (Dharmacon) and 16E6/E7 (pool of 3 siRNAs, Eurofins) at a final concentration of 50nM, using Lipofectamine RNAMaxi (Life Sciences Technologies, #13778-150). After 72 hours' incubation, cells were harvested and analyzed by western blotting.

18E6/E7 siRNA sequence: 5’ CAUUUACCAGCCCGACGAG 3’

16E6/E7 siRNA sequences: 5’ CACCUACAUUGCAUGAAU 3’

5’ CAACUGAUCUCUACUGUU 3’

5’ CCGGACAGAGCCCAUUAC 3’

Production and purification of GST-fusion proteins

The appropriate expression plasmids were transformed into E. coli strain DH5-α. The clones harbouring plasmids were grown in 40ml of Luria Broth (LB) culture media containing 75μg/ml Ampicillin (Sigma) overnight at 37°C. The overnight grown cultures were then transferred into 400ml of LB culture media containing 75μg/ml Ampicillin and incubated at 37°C for 1h. Isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1nM was added to induce recombinant protein expression and the culture was incubated for approximately 3h at 37°C in a shaker. Post IPTG treatment, the bacteria were harvested by centrifugation at 5000 rpm for 5 minutes. The bacterial pellets were lysed in 5-10ml of 1X PBS containing 1% Triton X- 100, and sonicated once/twice for 30 seconds at 80% amplitude. The lysates were then centrifuged again at 10000 rpm for 15 minutes. The supernatants were collected and incubated with glutathione-conjugated agarose beads on a rotating wheel overnight at 4°C. The GST-fusion protein-containing beads were then centrifuged at 2000 rpm for 1 minute and the supernatant was discarded. The beads were washed thrice with 1X PBS containing 1% Triton X-100. The GST-fusion protein-containing beads were then stored with 20% glycerol at -20°C.

GST pull-down and immunoprecipitation assays

HEK293 E6AP knockout cells were transfected with the appropriate plasmids and then harvested after 48 hours in RGMT lysis buffer (50mM HEPES pH 7.4, 150mM NaCl, 1mM $MgCl_2$, 1% Triton-x-100), supplemented with protease inhibitor cocktail I (Millipore, #539131). Cellular extracts were incubated with GST fusion proteins immobilised on glutathione agarose for 2 hours at room temperature. After exhaustive washes, bound proteins were detected by Western blotting.

To perform the co-immunoprecipitation assay for FBXO4 and E6 interaction, HEK293 E6AP K/O cells stably expressing GFP and GFP-18E6 were transfected with Flag-tagged FBXO4 plasmid. After 48 hours, proteasome inhibitor CBZ (20nM concentration) was added to each sample for 5 hours. After this incubation period, cells were harvested in RGMT lysis buffer. Cell lysates were first incubated with rabbit polyclonal anti-GFP antibody (1µg concentration) overnight at 4°C followed by incubating the antibody-cell lysate mix with Protein G (Amersham biosciences, #17-0618-02) for 2 hours at room temperature. Beads were extensively washed and protein analysis was performed using western blotting.

To perform the co-immunoprecipitation assays for E7 and E6AP interaction, HEK293 E6AP knockout cells were transfected with Myc-E6AP plasmid and the C-terminal FLAG/HA-tagged pCMV HPV-16 E7 or HPV-18 E7 plasmids, or an empty pCMV vector. After 24h, the cells were harvested in RGMT lysis buffer. Cell lysates were incubated with EZview™ Red Anti-HA Affinity Gel (Sigma-Aldrich, #E6779) for 3h at 4°C. The beads were then washed four times and processed by Western blotting.

Ubiquitination assay

For ubiquitination assays, the relevant plasmids were transfected into E6AP-null HEK293 cells, and, after 24h, cells were lysed in RGMT lysis buffer for 1 hour on ice. The cell lysates were incubated with anti-FLAG-conjugated agarose beads (Sigma Aldrich, #F2426) overnight at 4°C, to pull down ubiquitin-conjugated protein. The beads were then washed thrice for 5 minutes with RGMT buffer. The bound proteins were eluted in 2X sample lysis buffer, and the polyubiquitinated E7 proteins were detected using Western blotting.

In vitro phosphorylation assays

In vitro phosphorylation of the 18 E6 GST-fusion proteins was carried out using a DNA PK protein kinase system (Promega, #V4106) in the presence of radioactively labelled [γ-^{32}P] ATP. The reaction was carried out according to the manufacturer's protocol. The samples were analyzed by SDS-PAGE and autoradiography.

Dual Luciferase Assay

H1299 cells were transfected with appropriate plasmids expressing the luciferase reporters, and cell lysates were collected 24h post-transfection in lysis buffer. The luciferase assay was performed using the dual-luciferase reporter assay system (Promega, #E1960)), according to the manufacturer's instructions. The firefly luciferase and *Renilla* luciferase readings were taken using a TD20/20 luminometer by Turner Designs.

Immunofluorescence

For immunofluorescence staining, HEK293 E6AP knockout cells were seeded on lysine-coated coverslips in a 6-well plate. After 24 hours, cells were transfected with plasmids expressing HPV-16 E7 (100ng), HPV-18 E7 (100ng), and E6AP WT (1µg), using the $CaCl_2$ transfection method. After 5 hours' incubation, the media was changed, then the cells were incubated for a further 24 hours. After this, cells were washed once with 1X PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature, then washed thrice with PBS. The cells were permeabilised with PBS containing 0.5% TritonX-100 for 10 minutes at room temperature. After permeabilization, cells were incubated with goat monoclonal anti-HA-tag antibody (1:500 dilution), and mouse monoclonal anti-E6AP antibody (1:500) for 2 hours at 37°C in a humidified chamber. Cells were washed thrice for 5 minutes with PBS and then incubated with secondary antibodies (Alexa Fluor 546-conjugated anti-goat and Alexa Fluor 647-conjugated anti-mouse, 1:600 dilution) for 30 minutes at 37°C in humidified chamber followed by three 5-minute washes with PBS. Cells were then stained with DAPI and mounted. Image acquisition was performed using a ZEISS 880 Airyscan microscope at 60X with a digital zoom of 2.5X, and image analysis was performed using ImageJ software.

RT-qPCR analysis

Hela and SiHa cells were transfected with siRNAs against E6AP, HPV-18 E6/E7, HPV-16 E6/E7 or luciferase. After 72h, cells were harvested, and RNA was extracted using the Tri reagent system (Sigma Aldrich, #T9424) and subject to reverse transcription using the Quantitect Reverse Transcription Kit (Qiagen, #205311), according to the manufacturer's protocol. qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659) using the CFX96 Real-time PCR Detection System (BIORAD). The primers for HPV-16 and HPV-18 E7 genes and GADPH have been described previously (Bordigoni, Motte et al. 2021): 5´-3', HPV16 E7 (Forward), TCAGAGGAGGAGGATGAAATAGA; HPV16 E7 (Reverse), GCACAACCGAAGCGTAGA; HPV18 E7 (Forward), AATTCCGGTTGACCTTCTATGT; HPV18 E7 (Reverse), GGCTGGTAAATGTTGATGAT; GADPH (Forward), GGACCTGACCTGCCGTCTAG; GADPH (Reverse), TAGCCCAGGATGCCCTTGAG.

The level of each viral gene was normalised to that of GADPH as an indicator of the total input RNA.

Half-life experiments

siRNA knockdown of E6AP and Luci (Control) was performed in HeLa and SiHa cells, followed by incubation for 72 hours (as described above). Before harvesting, cells were further treated with Cycloheximide (1:2000; Sigma Aldrich #C4859) for different periods of time, after which the cells were harvested, and samples were analysed using western blotting.

Statistical Analysis

All experiments were performed at least thrice, and data are shown as mean and standard deviation of the mean. Statistical significance was calculated using the GraphPad Prism software using the unpaired two-tailed Student's t-test. A p-value below 0.05 was considered statistically significant and throughout the p values have been defined as follows * $p < 0.05$, ** $p < 0.005$, *** $p<0.001$ while “ns” represents a non-significant p-value above 0.05.

For the quantification of protein levels from western blots, the films were scanned, and the band intensities were measured using ImageJ software. The final relative quantification values are the ratio of the net band to the net loading control bands of Tubulin, actinin and β-galactosidase.

Results

Section 1:

HPV E6 induces the degradation of Dlg and MAGI proteins independently of the E6AP ubiquitin ligase *in vivo*.

An important feature of the cancer-associated HPV E6 oncoprotein is its ability to target cellular proteins for ubiquitin-mediated degradation. It has been reported in previous studies that high-risk HPV E6s complex with the E6AP ubiquitin ligase in order to constitutively degrade the p53 tumor suppressor protein from the host cells (Scheffner, Huibregtse et al. 1994, Thomas, Pim et al. 1999). Apart from p53, E6 is also known to target various other PDZ-domain containing cellular proteins that may also play an essential role in mediating tumorigenesis in these cells (Thomas and Banks 2018). The role of E6AP in the degradation of many of these E6 substrates is still ambiguous because loss of E6AP also induces a loss of E6 expression (Tomaic, Pim et al. 2009), thus it is difficult to ascertain whether lack of substrate degradation is due to lack of E6 or loss of E6AP ligase activity. To overcome this problem, we generated an E6AP null cell line using CRISPR/CAS9 (Thatte and Banks 2017) in which we could then re-express wildtype E6AP and a catalytically inactive form (C833A) (Talis, Huibregtse et al. 1998, Tomaic, Pim et al. 2009). The novelty of this strategy is that the catalytically inactive E6AP will itself remain functionally inactive, but it will be able to stabilize the E6 protein, rendering E6 capable of performing any activities that are E6AP-independent. To test the efficiency of this strategy, we first performed the assay on the p53 tumour suppressor protein, whose degradation is known to be solely dependent on E6AP ubiquitin ligase (Massimi, Shai et al. 2008). For this, we co-transfected the HEK293 E6AP knockout cells with constructs ectopically expressing the Flag-tagged p53, with either HA-tagged HPV 16 or HPV 18 E6, together with wildtype and catalytically inactive E6AP. After 48 hours, we harvested the cells and used immunoblotting to analyse p53 protein levels. As seen in Figure 11, p53 was efficiently degraded in the presence of functionally active E6AP over the course of assay, however, no p53 degradation was observed when the catalytically non-functional E6AP was co-expressed with either HPV 16 or 18 E6, as expected and in agreement with the previous studies (Scheffner, Huibregtse et al. 1993). However, both wildtype and catalytically inactive E6AP were able to stabilize E6 (Tomaic, Pim et al. 2009).

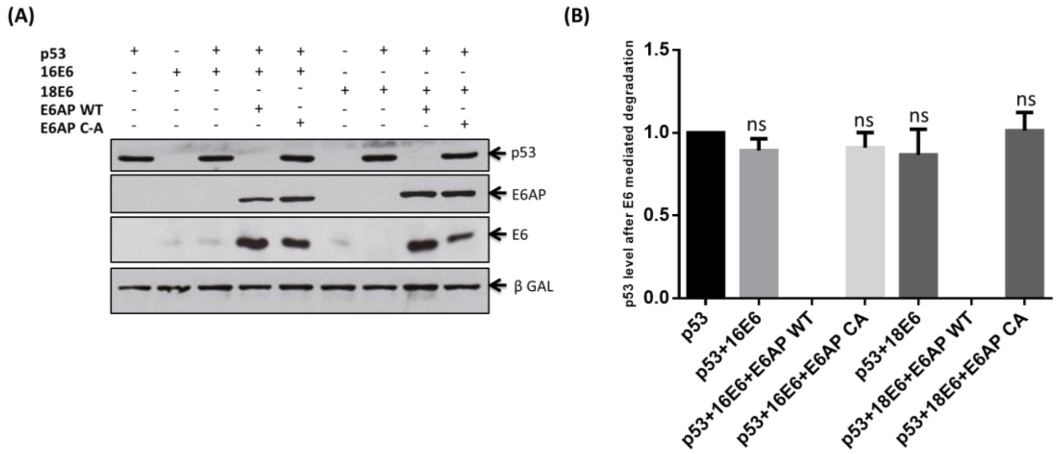


Figure 11. E6AP is required for E6-induced degradation of p53: (A) Cells were co-transfected with p53 expression plasmid, 1 ug Myc-tagged wild type E6AP and 1 ug Flag-tagged catalytically inactive E6AP expression constructs, together with 5 ug of the HA-tagged HPV-18 or HPV 16 E6 expression plasmids, as indicated, plus 100 ng of the LacZ expression plasmid, using calcium phosphate precipitation. After 48 hours, the cells were harvested and proteins were detected using western blotting with anti-Flag and anti-HA antibodies, followed by HRP-coupled anti-mouse antibody and HRP-conjugated anti-rabbit antibody. The blot was re-probed with an anti-β-galactosidase antibody to monitor protein loading and transfection efficiency. (B) Statistical quantification was done using Student's *t* test in the GraphPad Prism software. Values shown are means from at least 3 independent experiments; standard errors of the means are shown; ns represents not significant i.e. $p>0.05$ (Right panel).

Having confirmed that catalytically inactive E6AP could stabilize E6 and did not induce the degradation of p53, we proceeded to perform the same assay with some of the PDZ domain-containing targets of E6. We first investigated the MAGI family of proteins, which includes MAGI-1 and MAGI-3. Following the same protocol as for p53, we co-transfected the HEK293 E6AP knockout cells with plasmids expressing FLAG-tagged MAGI-1 and V5-tagged MAGI-3 in the presence of HPV-16 or HPV-18 E6, together with wildtype or catalytically inactive E6AP. Again, after 48 hours we harvested the cells and performed western blots to examine any changes in MAGI-1 and MAGI-3 protein levels. As can be seen in Figure 12 and 13 (A&B), we observed that MAGI-1 and MAGI-3 are degraded completely by both HPV 16 E6 and HPV 18 E6 in the presence of wildtype E6AP, as expected and has been previously shown. More interestingly, however, we observed that both MAGI-1 and MAGI-3 proteins disappeared almost completely in the presence of HPV-16 E6, and showed a significant decrease in the presence of HPV-18 E6, when expressed with catalytically inactive E6AP. E6 + MAGI-1 and E6 + MAGI-3 also exhibited a modest but significant reduction in the levels of MAGI-1 as well as MAGI-3, although the degree of degradation was different for different HPV types. Taken together, these results show that HPV-16 and HPV-18 E6-mediated degradation of both MAGI-1 and MAGI-3 is independent of E6AP, correlating with previously published *in vitro* data (Grm and Banks 2004, Massimi, Shai et al. 2008).

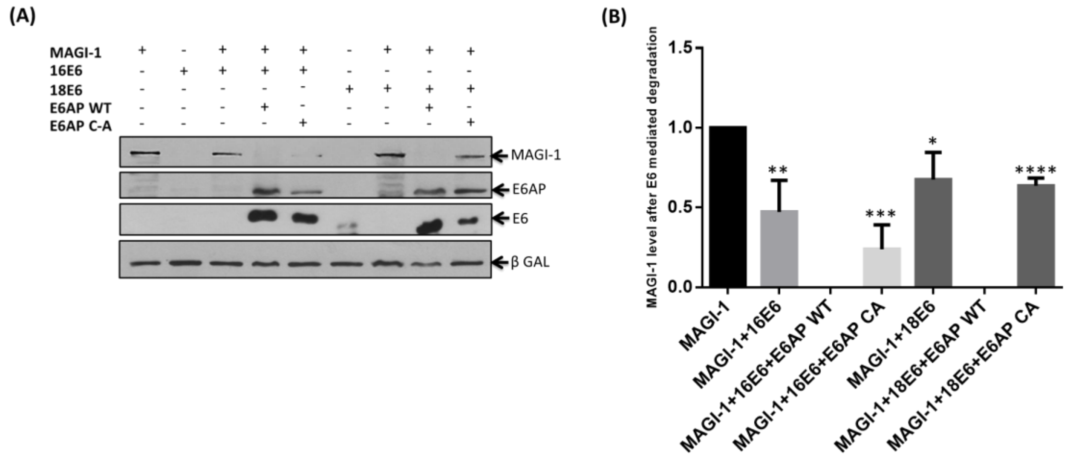


Figure 12. MAGI-1 degradation is induced by E6 independently of E6AP: HEK293 E6AP knockout cells were co-transfected with plasmid expressing FLAG-tagged MAGI-1 (100ng) together with those expressing either Myc-tagged wildtype E6AP (E6AP WT) or FLAG-tagged catalytically inactive E6AP (E6AP CA) (1µg each), in the presence of HPV-16 E6 or HPV-18 E6 (4µg each). Cells were harvested after 48 hours and proteins were analysed by western blot using antibodies specific to the tags. β -galactosidase was used as a transfection efficiency control (Left panel). Statistical analysis was performed using Student’s *t* test in the GraphPad Prism software. Values shown are the means from at least 3 independent experiments; standard errors of the

means are shown in the graph. ** and * represent p value<0.05, *** represents p value<0.005, **** represents p<0.0001 (Right Panel).

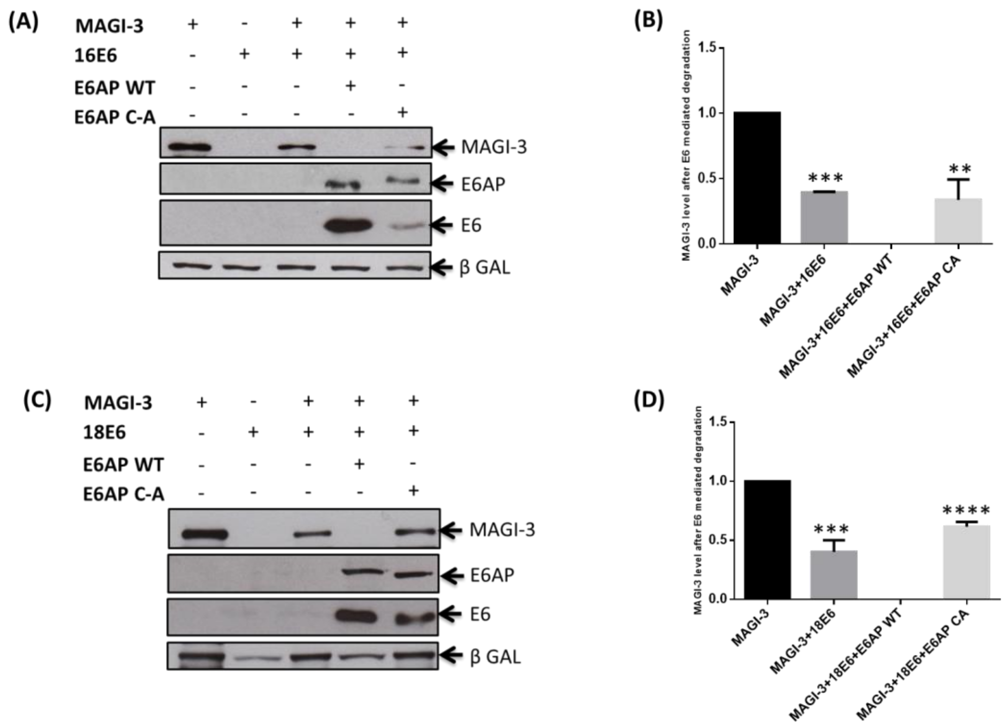


Figure 13. MAGI-3 degradation is induced by E6 independently of E6AP: HEK 293 E6AP knockout cells were co-transfected with plasmid expressing V5-tagged MAGI-3 (1µg) together with those expressing the wild type or catalytically inactive E6AP (1µg each), with either the HA-tagged HPV-16 (A) or HPV-18 E6 (C) (4µg each) expression plasmids. After 48 hours, cells were harvested, and proteins were analysed by western blot using tag-specific antibodies. Anti-β-galactosidase antibody was used for protein loading and transfection efficiency control. (B&D) Each column represents the mean value obtained from at least 3 independent experiments using Student's *t* test. Error bars represent Standard deviation. ** and *** represent p value<0.05 and **** represents p<0.0001.

We were next interested in ascertaining whether E6AP is required for E6-mediated degradation of the cellular polarity proteins DLG1 and Scribble (both components of the Scrib polarity complex), using the same assay. As can be seen in Figure 14, DLG1 levels markedly decrease when it is co-expressed with the catalytically inactive E6AP, and completely disappear in the presence of wildtype E6AP, as expected, in the case of both HPV-16 or HPV-18 E6. Whilst E6 + DLG1 also shows a decrease in the levels of

DLG, as for MAGI-1 and MAGI-3, clearly indicating the involvement of another pathway, independent of E6AP, in the E6-mediated degradation of DLG1 protein, corroborating previous studies. However, in the case of Scribble, no degradation was seen in the presence of catalytically inactive E6AP, as shown in Figure 15, suggesting that E6-directed degradation of Scribble is completely dependent on E6AP.

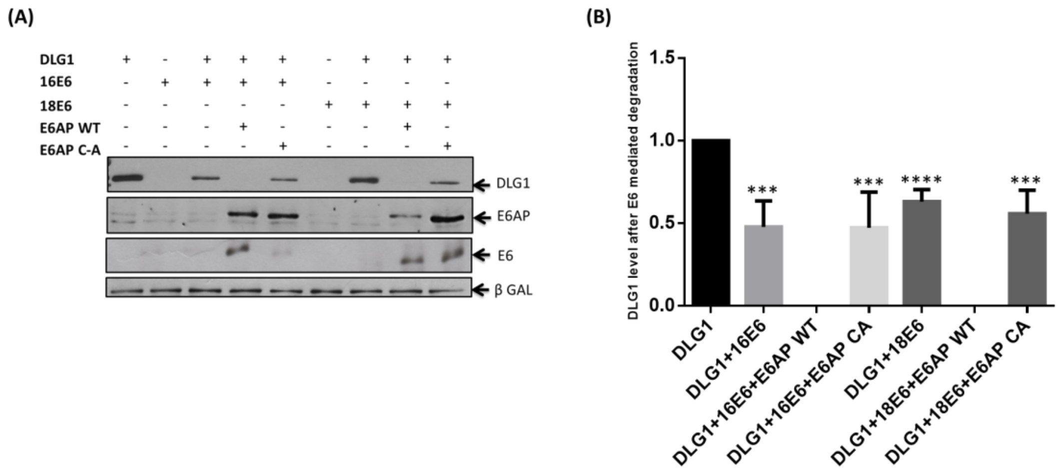


Figure 14. E6-mediated degradation of DLG1 is E6AP-independent: (A) HEK293 E6AP knockout cells were transfected with HA-tagged DLG1 expressing plasmid (1µg), together with plasmids expressing either wildtype or catalytically inactive E6AP (1µg each), and either HPV-16 or HPV-18 E6 (4µg each). After 48 hours, the cells were harvested and proteins were analysed by western blot, using tag-specific antibodies. β-galactosidase acted as a control for transfection efficiency. (B) Statistical analysis was performed using Student's *t* test from at least 3 independent experiments. Error bar represents the standard deviation. *** represents p value<0.001, **** represents p value< 0.0001.

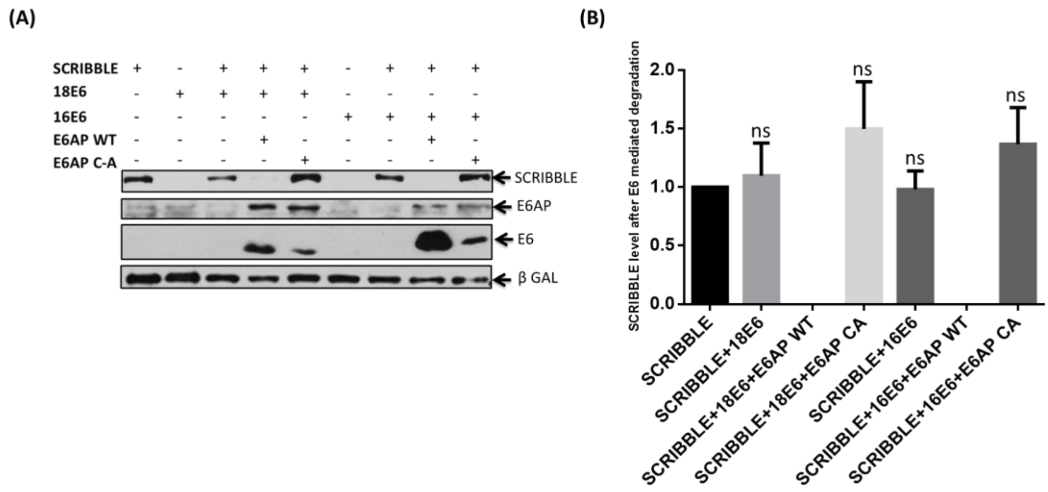


Figure 15. E6-directed degradation of Scribble is strictly E6AP-dependent: HEK293 E6AP knockout cells were co-transfected with HA-tagged Scribble-expressing plasmid (100ng), together with those expressing either wildtype or catalytically inactive E6AP (1µg each), and either HPV-16 or HPV-18 E6 (4µg each). After 48 hours, the cells were harvested and proteins were analysed by western blot, using tag-specific antibodies. β-galactosidase was used to monitor protein loading and transfection efficiency (Left panel). Statistical quantification was performed using Student's *t* test from at least 3 independent experiments. Error bars denote the standard deviation; ns represents non-significant with p value >0.005 (Right panel).

In order to confirm that the degradation of MAGI-1 in the presence of catalytically inactive E6AP was proteasome mediated we performed a MAGI-1 degradation assay in E6AP-knockout HEK293 cells with HPV-18 E6 protein in the presence of N-CBZ (Z-Leu-Leu-Leu-al, proteasome inhibitor) or NH_4Cl (Lysosomal protease inhibitor). As shown in Figure 16, in untreated samples, MAGI-1 levels are reduced significantly by HPV-18 E6 in the presence of catalytically inactive E6AP and this degradation is inhibited in the presence of proteasome inhibitor N-CBZ, rescuing MAGI-1 protein levels. However, no change was seen in MAGI-1 expression levels in samples treated with NH_4Cl, confirming the involvement of the proteasome degradation pathway. Accumulation of the LC3-II protein, which associates with autophagic membranes, serves as a positive control for the NH_4Cl samples (Riedel, Goldbaum et al. 2010).

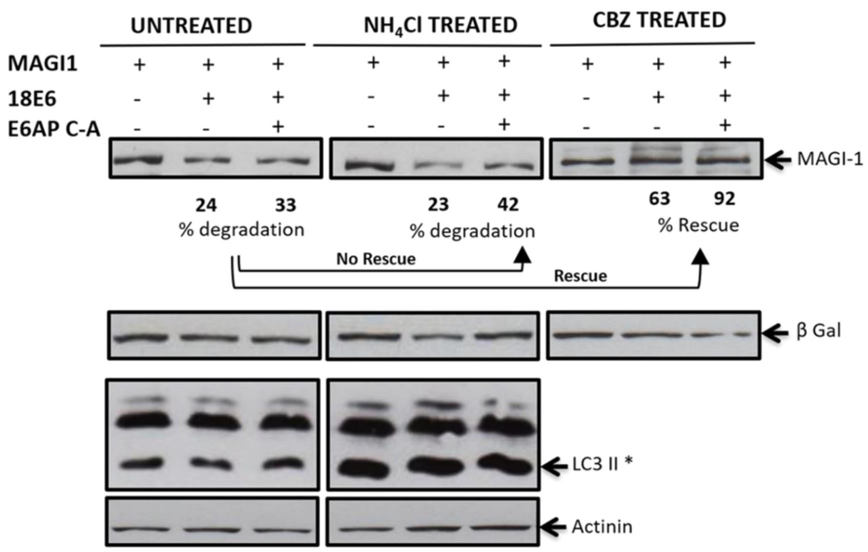


Figure 16. The proteasome inhibitor CBZ inhibits E6-directed degradation of MAGI-1 in vivo: HEK293 E6AP knockout cells were co-transfected with plasmids expressing FLAG-tagged MAGI-1 (100ng), HA-tagged HPV-18 E6 (4µg) and FLAG-tagged catalytically inactive E6AP (1µg). After 48 hours, CBZ (2µM) and NH_4Cl (25mM) were added to the respective samples, followed by further incubation for 5 hours. Cells were harvested and protein levels were detected using immunoblotting as indicated. β-galactosidase was used as a loading and transfection efficiency control, and LC3 was used as a positive control for NH_4Cl-treated samples, together with anti-α-actinin as its loading control.

It has been previously shown that interaction with E6AP stabilizes the HPV E6 oncoprotein by protecting it from proteasomal mediated degradation, possibly because the binding of E6 with E6AP masks the site of interaction of another possible ubiquitin ligase that might be involved in the degradation of E6 (Tomaic, Pim et al. 2009). So, next we decided to address this question by using the E6AP knockout cell line, as our study model. We first did a preliminary experiment in E6AP knockout cell lines by co-transfecting GFP-tagged 18E6, with either wildtype or catalytically inactive E6AP. 12 hours after transfection, we blocked the proteasomal degradation by treating the cells with CBZ, followed by incubation for a further 12 hours. We then harvested the cells and proteins expression analysis was performed using western blotting. As can be seen in Figure 17, in the sample expressing only GFP-tagged 18E6 without CBZ treatment, no GFP is seen, however, it is evidently rescued in the presence of CBZ, as expected.

We observed stable E6 protein levels in the presence of E6AP (WT and C-A), in the case of untreated samples (No CBZ), as well as in CBZ-treated samples, although more GFP-E6 was seen in CBZ-treated samples, in agreement with the previous studies (Tomaic, Pim et al. 2009).

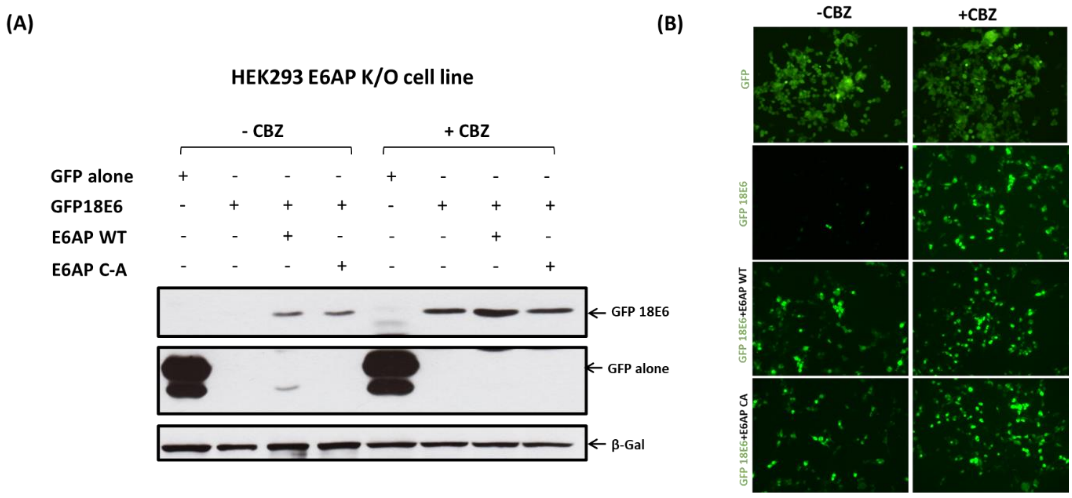


Figure 17. The proteasome inhibitor CBZ inhibits degradation of E6 oncoprotein: (A) HEK293 E6AP knockout cells were co-transfected with a GFP-Tagged 18E6 expressing plasmid (1µg), together with those expressing either wildtype or catalytically inactive E6AP (2µg each). After12 hours, the cells were treated with CBZ (2µM) and were incubated further for 12 hours. Cells were then harvested and probed for E6 protein expression levels by immunoblotting. β-galactosidase was used to monitor protein loading and as a transfection efficiency. (B) Represents the live-cell fluorescence images of the GFP-tagged 18E6, together with E6AP WT and C-A in the presence and absence of proteasome inhibitor CBZ. A plasmid expressing GFP was used as an experimental control, as shown in the figure.

Taken together, the above results confirm that another ubiquitin ligase may be involved in the E6-mediated degradation of PDZ domain-containing proteins, particularly Dlg and the MAGI family of proteins, and in the degradation of HPV E6 itself.

In order to identify the potential ligase involved in the degradation of HPV E6 and its cellular substrates, we next decided to perform High-Throughput siRNA library screening of all human ubiquitin ligases in the HEK293 E6AP knockout cell lines stably expressing GFP-tagged 18E6.

To generate the stable cell line, we first transfected HEK293 E6AP knockout cells with GFP-tagged 18E6 expressing plasmid. After 48 hours, we selected the cells with hygromycin at a concentration of 200ug/ml for 2 weeks, followed by isolation of single cell clones, as shown in Figure 18(A). These clones were then validated by confocal microscopy in the presence and absence of proteasome inhibitor CBZ, as shown in Figure 18(B).

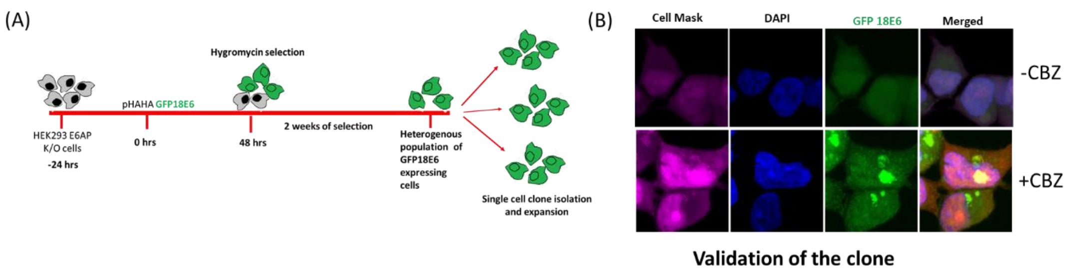


Figure 18. Generation and validation of 18E6-GFP tagged HEK293 E6AP knockout stable cell line. (A) Schematic diagram representing the generation of HEK293 E6AP knockout cells stably expressing the GFP-tagged 18E6 oncoprotein. (B) Represents the validation of the stable GFP-tagged 18E6-expressing single cell clone in the presence and absence of proteasome inhibitor CBZ.

After generating the desired clone stably expressing the GFP-tagged 18E6, we proceeded further to perform the siRNA library screen of the approximately 530 human ubiquitin ligases. To identify the ubiquitin ligase responsible for E6 and its target substrate degradation, we performed a high-content, fluorescence microscopy-based assay using a library of siRNAs against factors in the ubiquitin-conjugation system, including E1 and E2 enzymes and E3 RING and HECT domain ligases (598 target genes, 4 siRNAs per target, pooled). HEK293 E6AP knockout cells stably expressing GFP-tagged 18E6 were reverse-transfected in 384-well lysine coated plates with the pool of 4 different siRNAs targeting against each ubiquitin ligase (efficiency of transfection >85%). After 72 h, the cells were fixed, nuclei were counterstained with Hoechst 33342 and the whole cells were stained with cell mask (stains both cytoplasm as well as nuclei) and GFP fluorescence was analysed by high-content microscopy (workflow shown in Fig. 19).

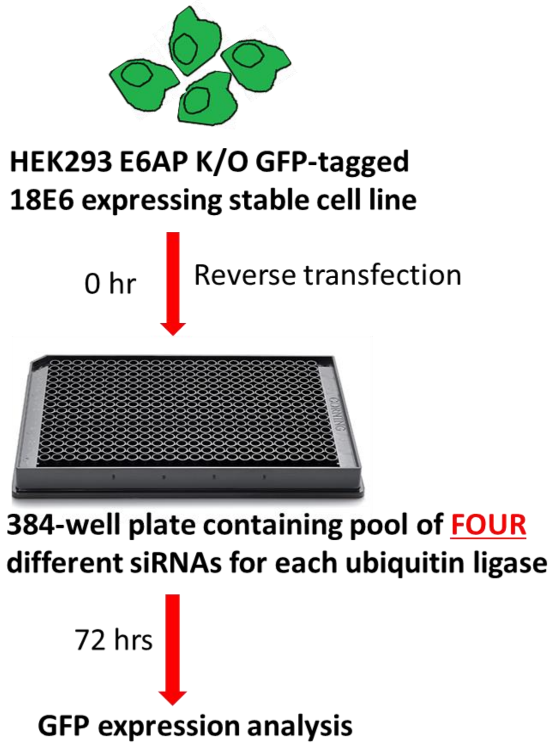
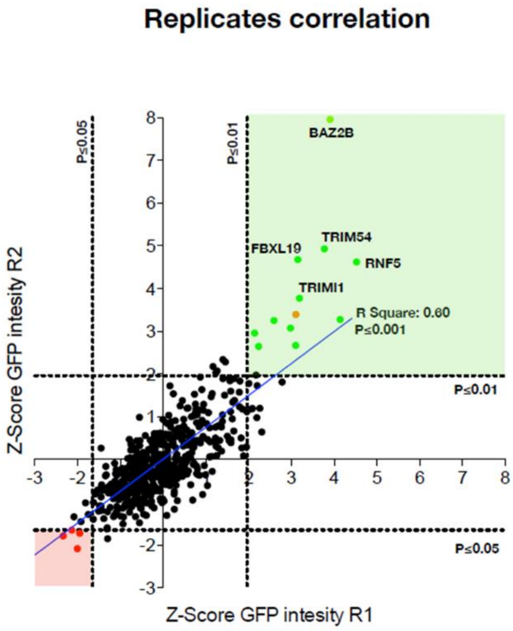


Figure 19. Workflow of siRNA Library Screening: A schematic diagram explaining the experimental outline of the high-throughput siRNA library screening of the human ubiquitin library screening in the HEK293 E6AP knockout cell line stably expressing GFP-tagged 18E6.

Two independent replicates of the screen were conducted; the replicates showed good reproducibility (Spearman $r = 0.60$; Fig. 20 A&B). The results of the two screens for the 598 siRNAs not impairing cell viability, were expressed as log10-fold over mock control (Figure 20). Treatment of the cells with proteasome inhibitor CBZ and with a non-targeting (NT) siRNA (both performed in quadruplicate) served as internal controls.



GENE NAME	R1 LOG2 FC	R1 Z score FC Intesity GFP	R2 LOG2 FC GFP Intensity	R2 Z score FC Intesity
BAZ2B	0.88317677	3.90551394	1.36772985	7.95434953
TRIM54	0.86292624	3.76902029	1.01140333	4.92710087
TRIMI1	0.76727678	3.14959376	0.97741089	4.67548376
RNF5	0.97139597	4.52300333	0.96976166	4.61967549
FBXL19	0.77344894	3.18833712	0.8483183	3.77215206
POS CTRL	0.75948836	3.10094084	0.79021188	3.39119307
CUL4B	0.91711215	4.13858638	0.77227588	3.27666616
RNF126	0.67530245	2.59147356	0.76860567	3.25340567
RNF144B	0.73921662	2.97553154	0.74023792	3.07560359
TRIM69	0.59643072	2.14039927	0.72155366	2.96039043
FBX04	0.75924581	3.09942988	0.67333702	2.66987415
UBe2QL1	0.6129088	2.23261188	0.66949334	2.64713025
ZNRF3	-0.5013649	-2.1363687	-0.3433019	-1.6531964
MEX3C	-0.4314184	-1.9500447	-0.3691306	-1.7281647
ASB1	-0.580136	-2.3356637	-0.3892996	-1.7857795
ZNF547	-0.4527422	-2.0078074	-0.498907	-2.0851833

Courtesy -Luca Braga

Figure 20. High-throughput siRNA-based screen to identify the ubiquitin ligase involved in the degradation of HPV 18E6 oncoprotein. (A) Results of screen: the graphs show the log10 values of the fold change of GFP-positive cells over control in the two replicate screenings (R1 and R2). The dotted lines show 2x increase over Control (pool of results using 4 non-targeting siRNAs and mock-transfected cells). (B) The 11 siRNAs in green are those that were the leading siRNAs in both the rounds of screening, showing an effect ≥2 fold over control. The effect of CBZ is shown in yellow. The 4 siRNAs in red are those that showed a decrease in the expression levels of 18E6 GFP upon their knockdown.

Upon analysis we observed that ablation of RNF5, BAZ2B, TRIM54, TRIML1 and FBXL19 resulted in a dramatic increase in the protein levels of the GFP-tagged 18E6, while ablation of CUL4B, RNF126, RNF144B, TRIM69, FBXO4 and UBE2QL1 gave a modest increase in the protein levels of GFP-tagged 18E6, as shown in Figure 20 and Figure 21.

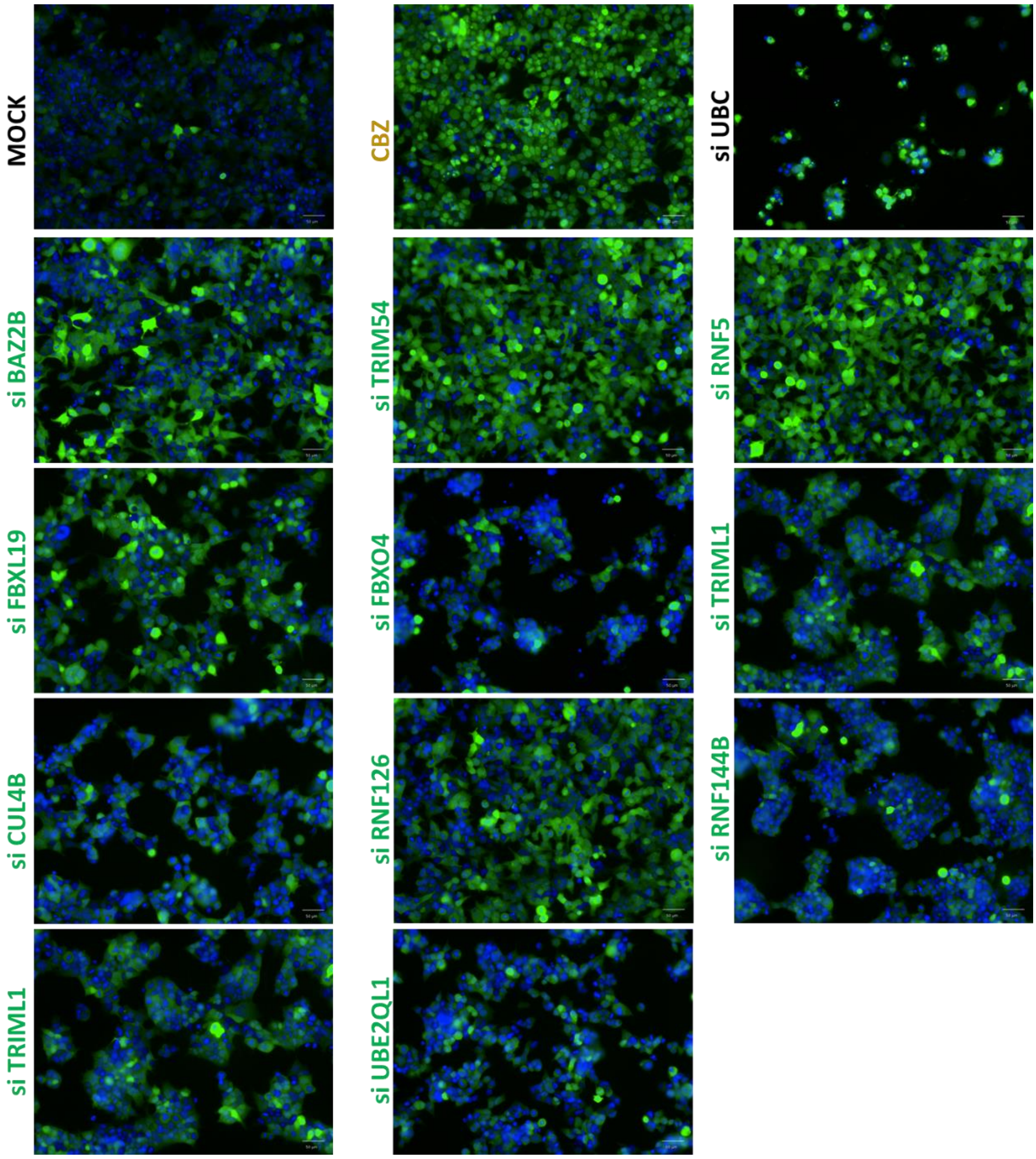


Figure 21. Representative high-content microscopy images showing increase in GFP-tagged 18E6 protein levels after depletion of the top 10 cellular ubiquitin-conjugation factors from the screens (shown in green), compared with Mock, which represents the untreated control cells with basal 18E6 GFP protein levels in HEK293 E6AP K/O cells, CBZ treated cells are the experimental positive control and siUBC (Ubiquitin C gene) is the transfection efficiency control. Scale bar is 50µm.

Intriguingly, we also found four ubiquitin ligases, ASB1, MEX3C, ZNF547 and ZNRF3, that, when silenced from the cells, led to a dramatic decrease in the protein expression patterns of GFP-tagged 18E6 as shown in Figure 22.

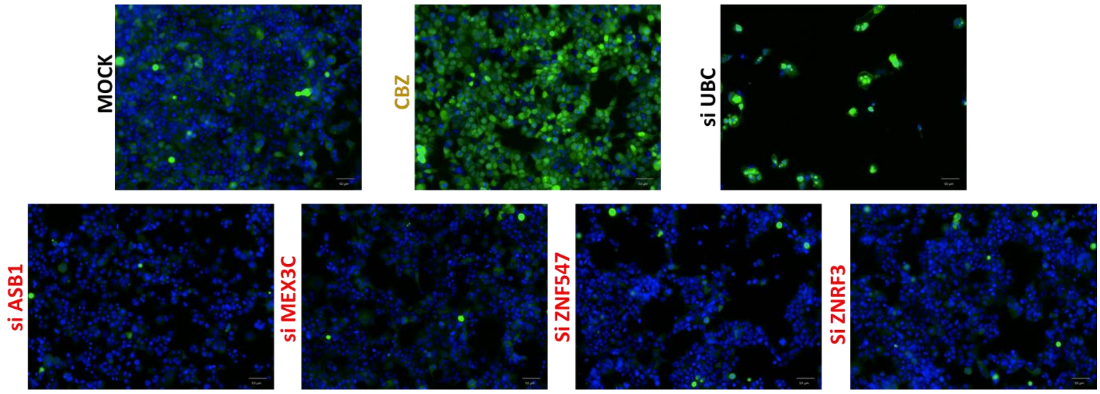


Figure 22. Representative high-content microscopy images showing decrease in GFP-tagged 18E6 protein levels after depletion of 4 cellular ubiquitin-conjugation factors (shown in red), compared with Mock, which represents the untreated control cells with basal 18E6 GFP protein levels in HEK293 E6AP K/O cells, CBZ treated cells are the experimental positive control and siUBC is the transfection efficiency control. Scale bar is 50µm.

Since the screen was performed in HEK293 E6AP K/O cells, which are derived from Human Embryonic Kidney, we decided to validate the 10 proteins identified by the screen in the cervical cancer-derived cell line, HeLa. To do this, we reverse-transfected HeLa cells with the siRNAs against each ubiquitin ligase, together with siE6AP and si Luci (luciferase) as control. The results obtained are shown in Figure 23; as expected, knockdown of E6AP reduces the levels of E6, in agreement with previous studies (Tomaic, Pim et al. 2009), when compared with the control Luci. Interestingly, upon analysis, we observed that only knockdown of FBXL19, FBXO4 and TRIM69, together with E6AP, showed any significant rescue of 18E6 protein levels, while siBAZ2B+E6AP and siCUL4B+E6AP showed a modest increase in 18E6 protein levels, when compared with siE6AP. To our surprise, knockdown of the other proteins identified in the screen (together with E6AP), including RNF5, TRIM54, UBE2QL1, RNF144B and RNF126, did not have any effect on the levels of 18E6.

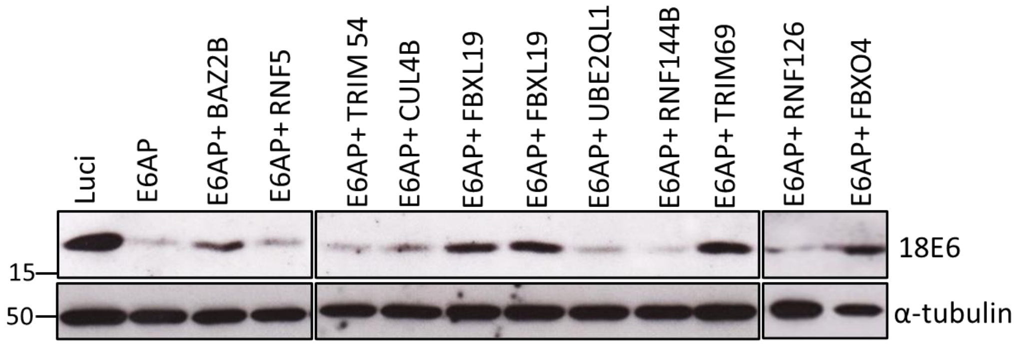


Figure 23. Validation in HeLa cells of the proteins identified by the library screening: HeLa cells were reverse-transfected with siRNAs targeting each identified protein, together with siE6AP and siLuciferase (Luci) as control. After 72 hours, cells were lysed and protein expression analysis was performed using immunoblotting; α-tubulin was used as a loading control.

Based on these results, we decided to focus on FBXO4, which is a substrate recognition component of SCF (SCF-Cul1-Fbox complex), an E3 ubiquitin ligase complex. It recognizes and mediates the ubiquitination and subsequent degradation of target proteins in a proteasome-dependent manner (Qie, Majumder et al. 2017). To begin with, we first decided to perform an *in vivo* degradation assay of E6 in the presence of FBXO4 in HEK293 E6AP K/O cells. To do this, we co-transfected HEK293 E6AP K/O cells with plasmids expressing Flag-tagged FBXO4, GFP-tagged 18E6, Empty GFP (as control) and HA-tagged E6AP. After 48 hours, cells were harvested and protein analysis was performed using immunoblotting. As can be seen in Figure 24A, GFP-tagged 18E6 undergoes degradation in the presence of FBXO4, whereas in the presence of E6AP+FBXO4, the GFP-tagged 18E6 is stabilised. Control GFP remains unaffected in the presence of either FBXO4 alone or E6AP+FBXO4, suggesting that the effect observed is specific to 18E6 and not to the GFP tag. Taken together, these results suggest that FBXO4 targets the degradation of E6 in the absence of E6AP, as transfecting E6AP back into the cells rescues the levels of E6 oncoprotein.

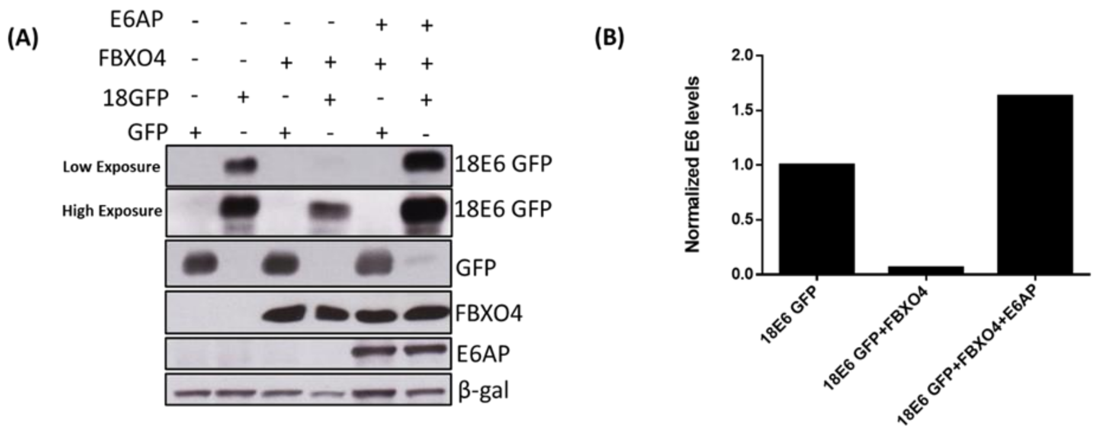


Figure 24. FBXO4 degrades HPV 18E6 in the absence of E6AP. (A) HEK293 E6APK/O cells were transfected with plasmids expressing empty GFP (500ng), GFP-tagged 18E6 (500ng), Flag-tagged FBXO4 (4µg), HA-tagged E6AP (4µg) and β-gal (100ng). After 48 hours, proteins were extracted and immunoblotting was performed. E6 was detected using antibody specific to GFP; FBXO4 and E6AP were detected using antibodies to the endogenous proteins. β-gal was used as a transfection loading control. (B) Represents the histogram showing quantification of the E6 oncoprotein with the values being normalised with β-gal.

We next proceeded to validate the results obtained from the *in vivo* degradation assays in HPV-18 positive cervical cancer-derived cell lines, HeLa and C41. To do this, we reverse-transfected HeLa and C41 with the siRNAs against E6AP, FBXO4 and Luci as control. After 72 hours, cells were harvested and protein expression analysis was performed using immunoblotting. As can be seen in Figure 25, siE6AP reduces the levels of E6 oncoprotein in both HeLa and C41 cell lines, as expected and in agreement with previous studies (Tomaic, Pim et al. 2009), whereas siFBXO4 has no obvious effect on the levels of E6 oncoprotein. However, silencing both FBXO4 and E6AP leads to a significant increase in the levels of E6 oncoprotein, both in HeLa and C41 cervical cancer cell lines. These results suggest that FBXO4 targets E6 for degradation in the absence of E6AP, as its knockdown with E6AP leads to stabilisation of E6 protein levels.

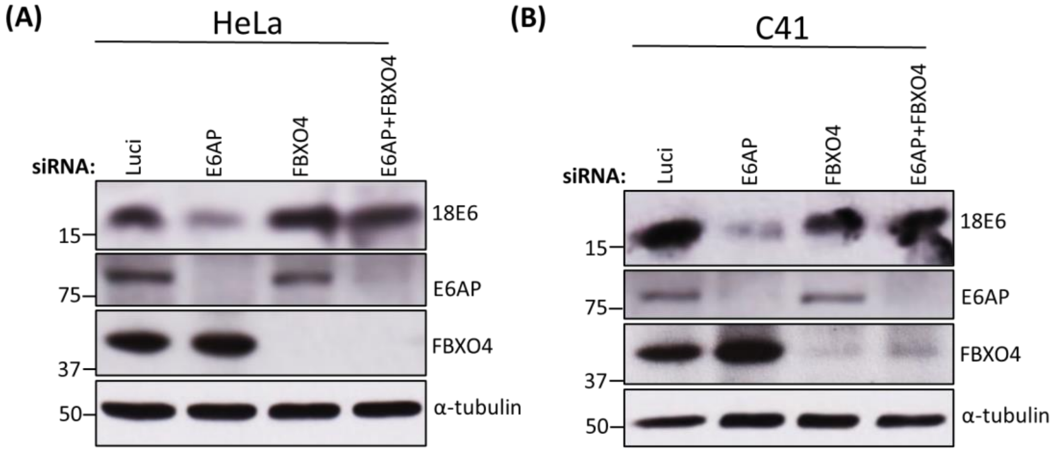


Figure 25. FBXO4 targets E6 in the absence of E6AP. (A) HeLa and (B) C41 cell lines were reverse-transfected with siRNAs against E6AP and FBXO4 (50nM each). siRNA against Luci was used as control. After 72 hours, cells were harvested and protein expression analysis was performed using western blotting for each protein, as indicated. α-tubulin was used as a loading control.

FBXO4 interacts with HPV E6 oncoprotein

We next wanted to ascertain if FBXO4 interacts with the HPV E6 oncoprotein. To determine this, we performed an *in vitro* GST pull-down assay using GST-tagged HPV-16 and 18 E6. We first transfected the HEK293 E6AP K/O cells with the plasmid expressing FBXO4. After 48 hours, cells were lysed and the cellular extract was incubated overnight at 4°C with GST-tagged HPV-16 and HPV-18 E6, and empty GST as negative control, followed by immunoblot analysis. As can be seen in Figure 26(A), both HPV-16 and HPV-18 E6 interact with FBXO4. We further validated this *in vivo* by performing a co-immunoprecipitation assay. To do so, we transfected the plasmid expressing FBXO4 into HEK293 E6AP K/O stable cell lines expressing GFP alone and GFP-tagged 18E6. After 48 hours, cells were treated with proteasome inhibitor CBZ at a concentration of 20nM for 5 hours. Cell extracts were isolated and incubated with anti-GFP antibody followed by co-immunoprecipitation on agarose beads. The results obtained are shown in Figure 26(B), where it can be seen that GFP-tagged 18E6 interacts with FBXO4, while the control GFP does not, further confirming the interaction of 18E6 with FBXO4.

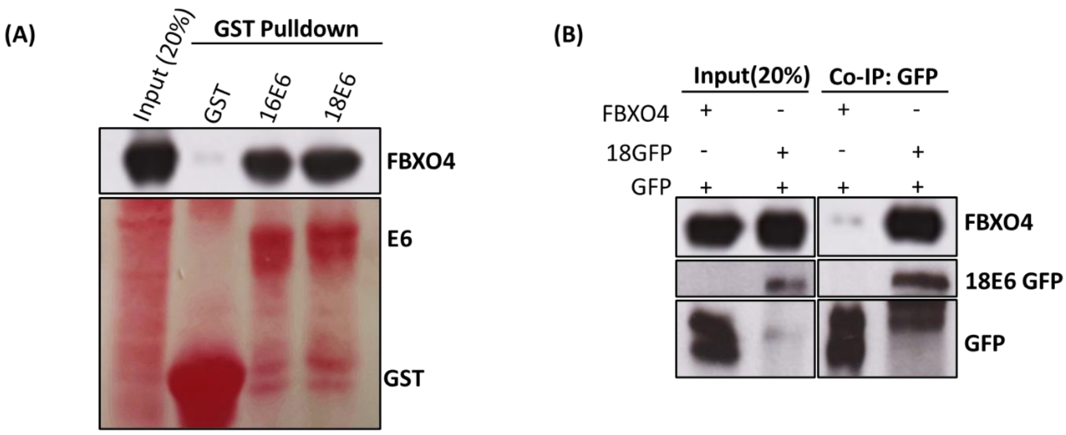


Figure 26. FBXO4 interacts with E6 oncoprotein: **(A)** GST pull-down assays were performed using lysates of HEK293 E6AP K/O cells ectopically expressing Flag-tagged FBXO4 (5µg), together with purified GST-tagged HPV-16 E6, GST-tagged HPV-18 E6 and empty GST as control. The top panels show the immunoblot analysis for FBXO4 protein probed using anti-FBXO4 antibody, and the lower panels show the Ponceau stain for different GST fusion proteins. **(B)** HEK293 E6AP K/O cells stably expressing empty GFP and GFP-tagged 18E6 were transfected with plasmid expressing Flag-tagged FBXO4 (5µg). After 48 hours, cells were treated with CBZ (20nM), proteasome inhibitor and then after 5 hours, cell extracts were analysed by co-immunoprecipitation, using anti-GFP antibody immobilised on agarose beads. Protein analysis was performed using immunoblotting. FBXO4 was probed using anti-FBXO4 antibody; GFP-tagged 18E6 and empty GFP was probed using anti-GFP antibody.

The presence of E6AP reduces the interaction between E6 and FBXO4

After confirming the interaction of 18E6 with FBXO4 in the absence of E6AP, we next were interested in ascertaining if the presence of E6AP can perturb the interaction between 18E6 and FBXO4. To examine this, we repeated the co-immunoprecipitation assay in HEK293 E6AP K/O cells stably expressing empty GFP and GFP-tagged 18E6 and transiently expressing Flag-FBXO4 and myc-E6AP. As can be seen in Figure 27, as expected, both E6AP and FBXO4 bind to E6 when expressed individually with E6, but, interestingly, the interaction between FBXO4 and E6 is markedly reduced when E6AP is co-expressed, suggesting that E6AP may compete with FBXO4 to bind E6.

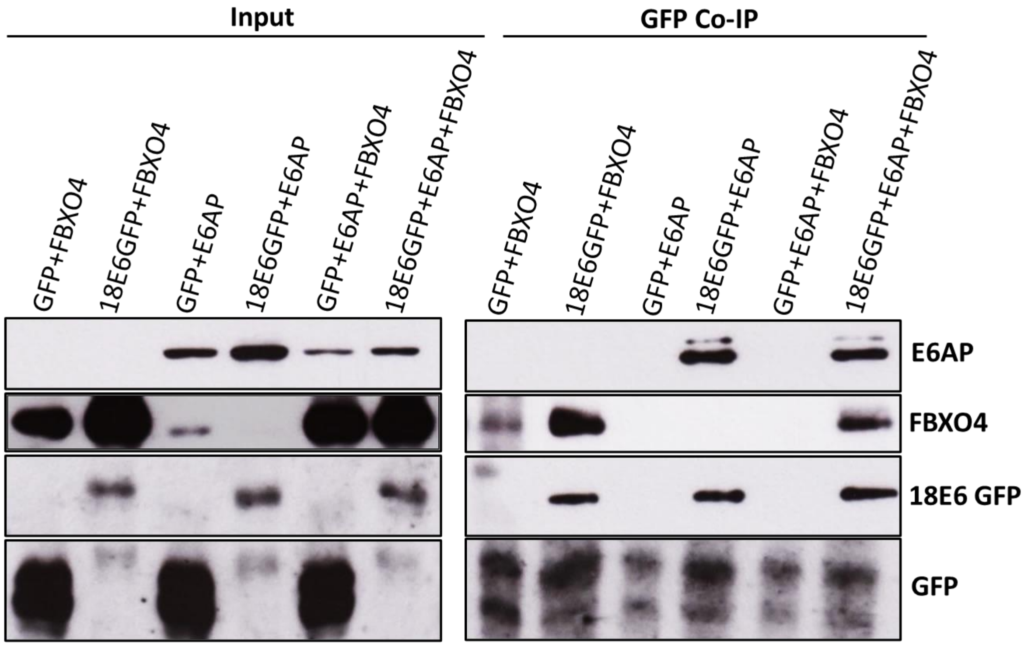


Figure 27. Interaction between FBXO4 and E6 is reduced in the presence of E6AP. HEK 293 E6AP K/O cells stably expressing GFP and 18E6 GFP were transfected with plasmids expressing Flag-FBXO4 (5μg) and HA-E6AP (5μg). After 48 hours, cells were treated with CBZ (20nM) for 5 hours, cellular proteins were isolated and co-immunoprecipitated using anti-GFP antibody, followed by analysis using western blotting. FBXO4 and E6AP were probed using endogenous antibody respectively; GFP and 18E6 GFP were probed using anti-GFP antibody.

Knockdown of FBXO4 and E6AP induces cell death in HPV-positive cervical cancer cells in a p53-dependent manner

During our studies in the cervical cancer-derived cell line HeLa, we observed that the silencing of E6AP together with FBXO4 not only rescues the protein levels of HPV 18E6 (Figure 28B), but also induces high levels of cell death, as can be seen in Figure 28A. This was surprising because we had expected that the rescue in E6 protein levels would lead to an increase in cell survival, but we saw the opposite result. In order to investigate a potential mechanism, with p53 being a strong candidate for inducing such apoptosis we repeated the knockdown of E6AP+FBXO4 but also included siRNA p53.

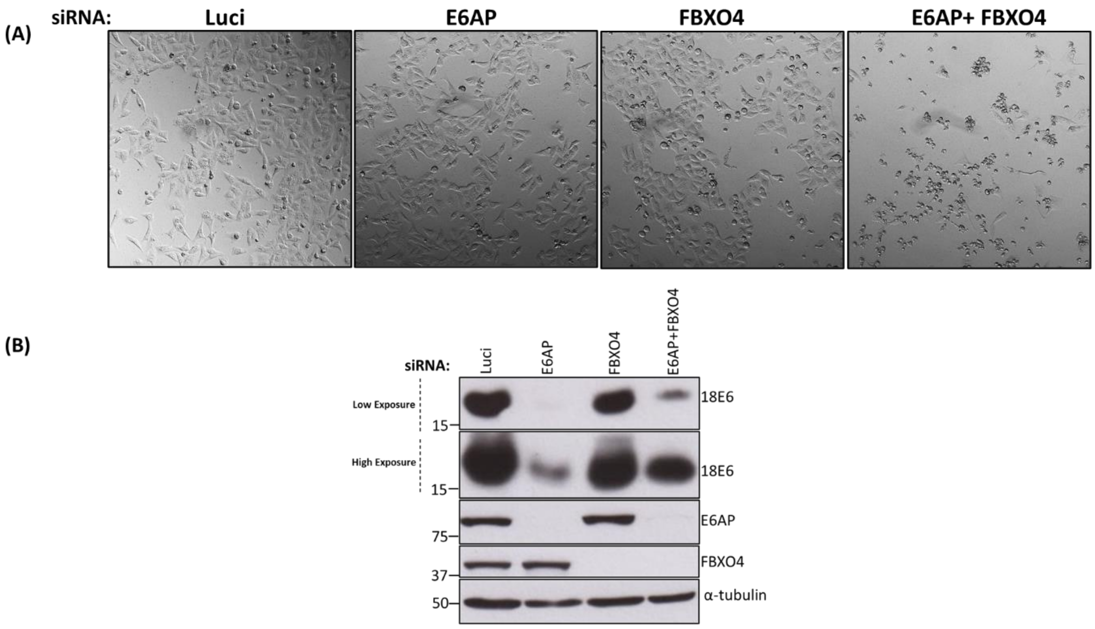


Figure 28. Ablation of E6AP+FBXO4 induces cell death in the cervical cancer-derived HeLa cell line. HeLa cells were reverse-transfected with siRNAs specific to E6AP, FBXO4 and Luci (control) at a final concentration of 50nM. After 72 hours, live cell images were taken (A) and the cells were then harvested followed by protein expression analysis using immunoblotting (B). 18E6, E6AP and FBXO4 were probed using the respective endogenous antibodies and α-tubulin was used as loading control.

As can be seen in Figure 29A, ablation of E6AP+FBXO4 induces a phenotype indicative of cell death, (also shown in Figure 28), but surprisingly this phenotype was reversed when we silenced p53 on top of E6AP+FBXO4. However, the protein levels of E6 remain unchanged in both conditions i.e. si E6AP+FBXO4 and si E6AP+FBXO4+p53, as can been seen in Figure 29B. Taken together, these results suggest that knockdown of E6AP+FBXO4 increases the E6 protein levels and induces cell death in a p53-dependent manner.

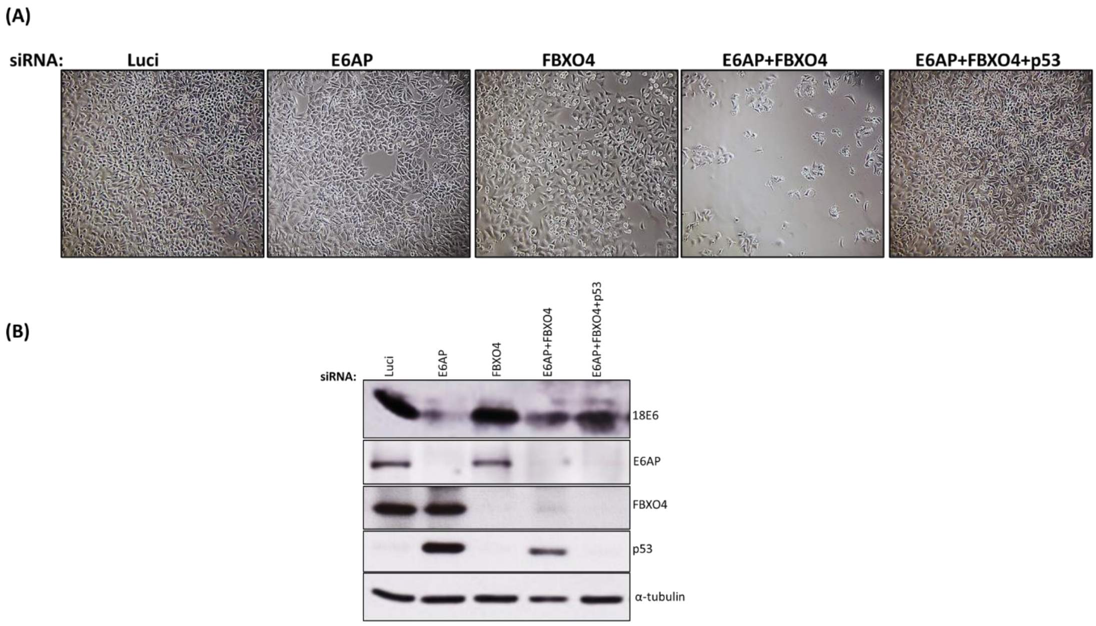


Figure 29. Knockdown of E6AP+FBXO4 leads to cell death in p53-dependent manner. HeLa cells were reverse transfected with the siRNAs against E6AP, FBXO4, p53 and Luci (control). After 72 hours, live cells images were taken as shown in top panel A, and then cellular extracts were prepared followed by western blot analysis, as shown in bottom panel B. 18E6, E6AP, p53 and FBXO4 were probed using respective endogenous antibodies and α-tubulin was used as loading control.

Having shown that the combined knockdown of E6AP and FBXO4 leads to dramatic cell death in a p53-dependent manner in the cervical cancer-derived cell line, HeLa, we next wanted to determine if this effect is specific to HPV-positive cervical cancer cells. To examine this, we decided to perform an XTT-based calorimetric assay to determine the cell viability in three different cell lines, including NIKS (normal immortalized keratinocytes), HeLa (HPV-18 positive cervical cancer cells) and C33A (HPV-negative cervical cancer cells). The principle of this assay is based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent, producing a soluble coloured formazan salt, associated with the cellular metabolic activity (Kuhn, Balkis et al. 2003). To perform this assay, we repeated the knockdown experiment as described earlier, but in a 96 well plate format, for all the three cell lines. After 72 hours, XTT-labelling reagent was added to each sample followed by incubation for 5 hours. After this incubation period, the formazan dye formed was quantitated using a scanning multi-well spectrophotometer (ELISA reader) at a wavelength of 490. The measured absorbance directly correlates with the number of viable cells. As can be seen in Figure 30, NIKS and C33A cell lines did not show significant effects on their cell viability upon silencing of either E6AP or FBXO4 individually or in combination. However, in the case of HeLa cells, silencing of E6AP and FBXO4 separately resulted in a slight decrease in the number of viable cells, when compared with the Luci control, but, interestingly, when both E6AP and FBXO4 were knocked down, this effect was more pronounced, with the cell viability reduced to about 25%. Additionally, silencing p53 together with E6AP+FBXO4 increased the cell viability to above 75% in HeLa cells, while E6AP+FBXO4 gave a cell viability of about 25%. NIKS and C33A cells showed no differences in their cell viability when p53+E6AP+FBXO4 were ablated together from these cells. These results demonstrate that the decrease in the cell viability upon silencing E6AP+FBXO4 is specific to HPV-positive cervical cancer cells and is dependent on the presence of p53.

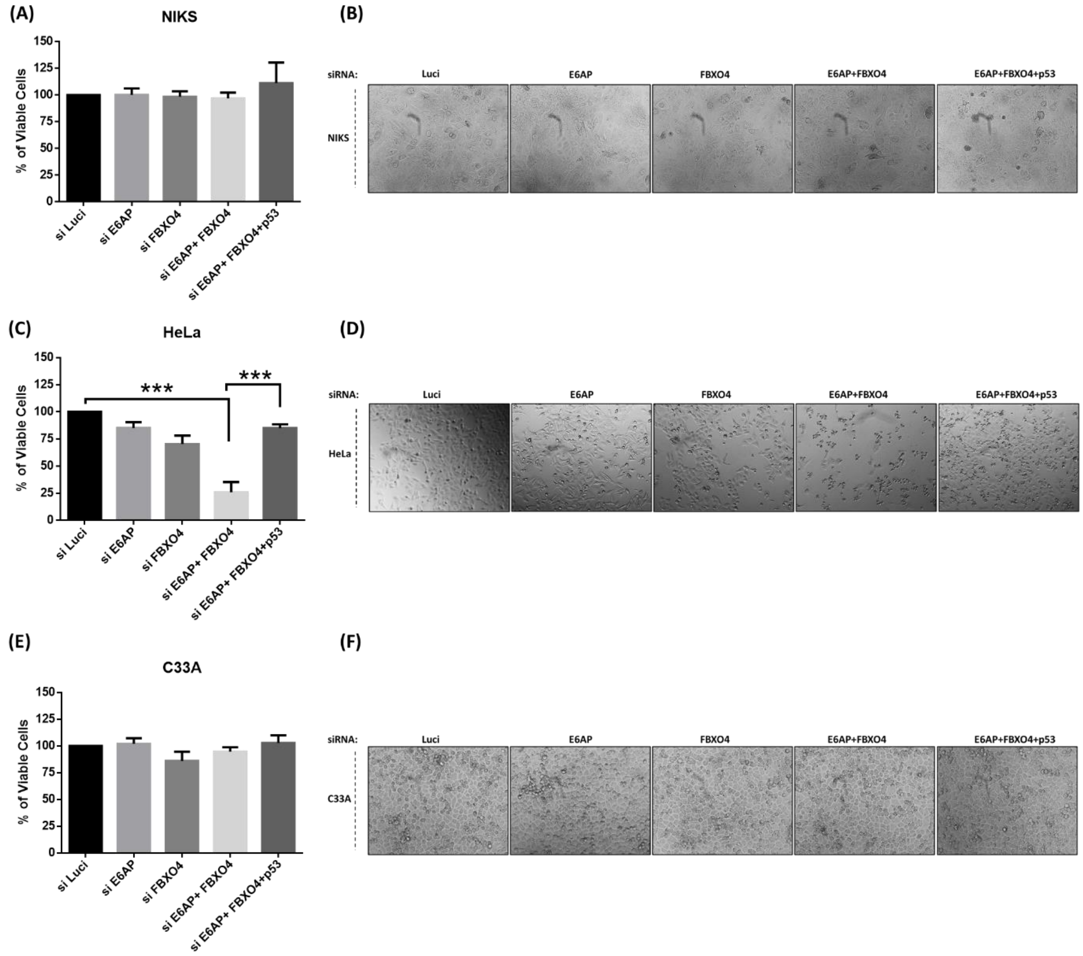
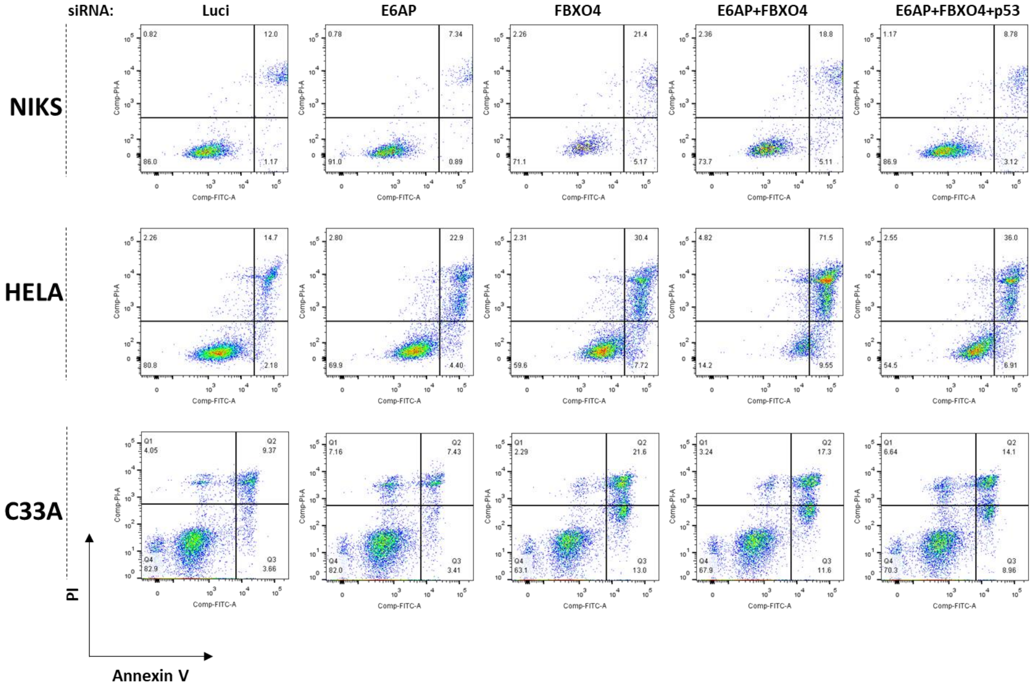


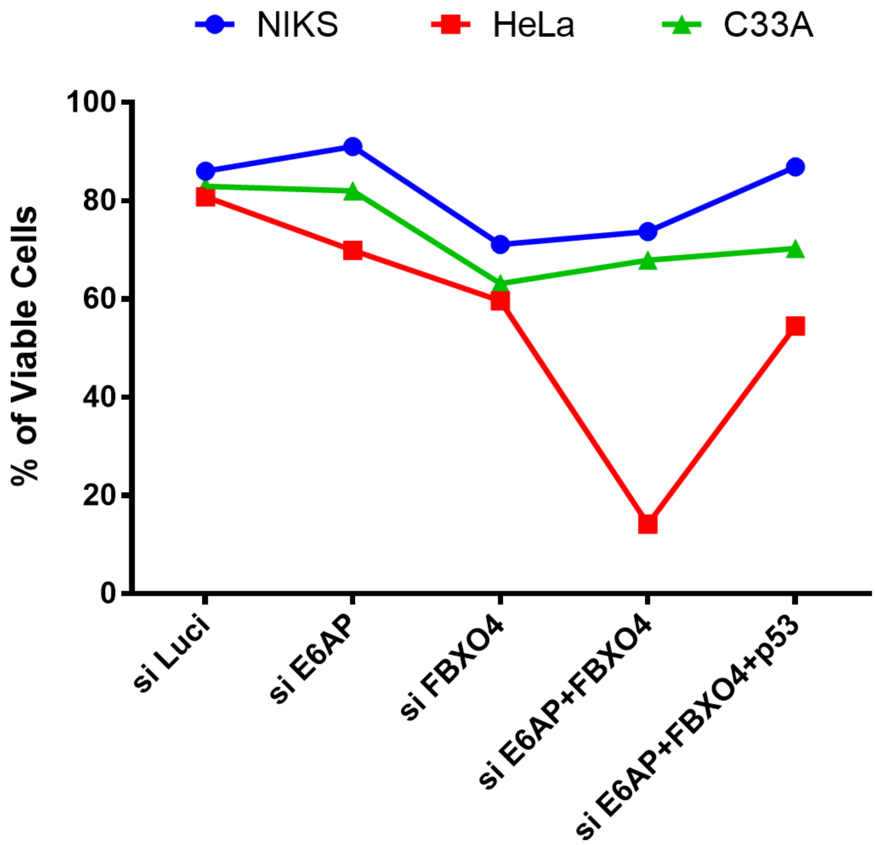
Figure 30. XTT-based cell viability assay in NIKS, HeLa and C33A cell lines: NIKS (A), HeLa (C), and C33A (E) cells were reverse transfected with the siRNAs as indicated in 96-well plate. After 72 hours, Live cells images were taken for each cell line (B, D & F) and then cells were treated with XTT-labelling reagent followed by further incubation for 5 hours. Cell viability was quantified using ELISA plate reader at wavelength of 490. Statistical analysis was performed for each histogram using Student's t-test. *** represents p-values<0.001; Error bars represent the standard deviation from at least 3 independent experiments.

Taking measurements from metabolic activity-directed tests remains a useful method of obtaining preliminary information, however, these tests have some limitations and have been shown to be highly susceptible to metabolic interference, hence they may not provide unambiguous results on cell viability (Kuhn, Balkis et al. 2003, Berridge, Herst et al. 2005). Therefore, we next decided to validate results obtained from the XTT-assay by apoptosis-based assays using the cell death markers Annexin-V and propidium iodide (PI). For this, we repeated the knockdown experiment described in previous experiments, and after 72 hours cells were collected and labelled with propidium iodide (PI) and Annexin-V FITC, followed by evaluation of the apoptotic process using fluorescence-activated cell sorting (FACS) analysis. The results obtained are shown in Figure 31. Upon analysis, we observed that the knockdown of E6AP from NIKS and C33A resulted in no differences in the percentage of viable cells, when compared with si Luci (control), whereas in HeLa cells, silencing of E6AP showed a modest decrease in the percentage of viable cells, as can be seen Figure 31 (A&B), as expected and in agreement with the previous studies (Hengstermann, D'Silva M et al. 2005). We also found that ablation of FBXO4 from all the cell lines gave a modest decrease in the percentage of viable cells, compared with control Luci, which could be possible as FBXO4 has been shown to be involved in regulation of cancer cell growth (Lee, Perrem et al. 2006). Intriguingly, when both E6AP and FBXO4 were silenced together, the percentage of viable cells in HeLa decreased dramatically to 14.2% compared with 80.8% viable cells in the Luci control, whereas there was no effect on the viability of NIKS and C33A in this condition. In addition, the triple knockdown of E6AP+FBXO4+p53 in HeLa cells significantly rescues the number of viable cells, when compared with siE6AP+FBXO4. Again, we observed no changes in the cell viability for the triple knockdown in NIKS or C33A cells. We validated the knockdown of all the proteins by western blotting, as can be seen in Figure 31C. Taken together with the previous observation, this result suggests that the decrease in the cell viability and increase in apoptosis upon silencing E6AP+FBXO4 is mediated by p53 and is specific to HPV-positive cancer cells.

(A)


siRNA:
Luci
E6AP
FBXO4
E6AP+FBXO4
E6AP+FBXO4+p53
NIKS
HELA
C33A
PI
Annexin V
Comp-FITC-A
Comp-PI-A

(B)


NIKS
HeLa
C33A
% of Viable Cells
100
80
60
40
20
0
si Luci
si E6AP
si FBXO4
si E6AP+FBXO4
si E6AP+FBXO4+p53

(C)

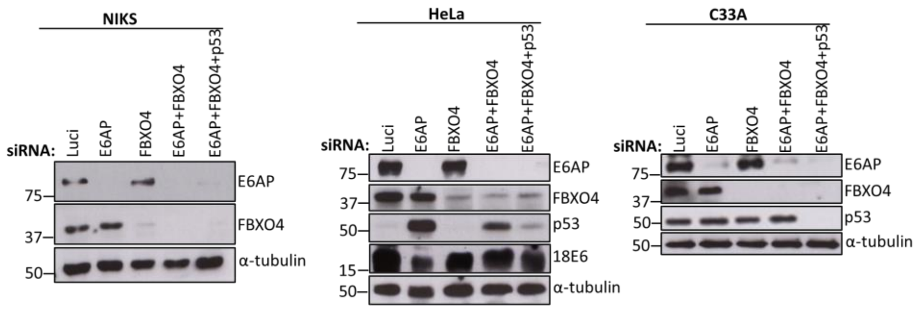


Figure 31. Representative dot plots of both Annexin V-FITC (X-axis) and PI-stained (Y-axis) NIKS cells HeLa and C33A cells. NIKS, HeLa and C33A cells were reverse transfected with siRNAs against Luci (control), E6AP, FBXO4 and p53 at a concentration of 50nM. After 72 hours, cells were collected followed by evaluation of apoptosis using FACS analysis (A). In the different quadrants the percentage of cells were reported: Viable cells, lower left quadrant (Q4); early apoptotic cells, bottom right quadrant (Q3); late apoptotic cells, top right quadrant (Q2); non-viable necrotic cells, upper left quadrant (Q1). Scatter dot blots were plotted using FlowJo software. (B) Represents the graph showing the patterns of the percentage of viable cells for NIKS (in blue), HeLa (in Red) and C33A (in green). (C) Represents the western blot analysis showing the knockdown of all the proteins; E6AP, FBXO4 and p53; E6 protein levels were probed in HeLa cells. α-actinin was used a loading control.

Section II

E6AP stabilizes the HPV E7 oncoprotein

During the course of the above studies, we included E7 as control in some of the initial assays on siE6AP in cervical cancer derived cells. We made the surprising observation when we found that knockdown of E6AP not only destabilizes E6 oncoprotein but also destabilizes E7 oncoprotein. A typical example of such an assay is shown in Figure 32, where knockdown of E6AP leads to a significant decrease in the protein levels of both HPV-16 and HPV-18 E7, compared with the Luci control. This result suggests that the presence of E6AP plays a crucial role in maintaining the levels of E7 oncoproteins of both HPV-16 and HPV-18, similar to its effect on the E6 oncoprotein (Tomaic, Pim et al. 2009).

We next wanted to ascertain if the ectopic expression of E6AP in HEK293 E6AP-knockout cells (HEK293 E6APK/O) could stabilise E7 protein levels. To test this, we co-transfected HEK293 E6APK/O cells with plasmids expressing E6AP, 18E7 and 18E6; after 24 hours the cells were harvested, and the protein expression patterns of both HPV-18 E7 and HPV-18 E6 were examined by western blotting. Interestingly, we observed that E7 protein levels were greatly increased in the presence of E6AP, compared with levels in the absence of E6AP, similar to the E6 oncoprotein. As can be seen in Figure 33A, we validated this blot using the respective endogenous antibodies raised against HPV-18 E6 and HPV-18 E7 and also verified this by using the tag-specific anti-HA antibody.

Having shown that the stability of high-risk HPV E7 is dependent on E6AP, we next wanted to ascertain if the presence of E6AP could also lead to stabilisation of low-risk HPV E7 proteins. To do this, we co-transfected the HEK293 E6APK/O cells with plasmids expressing high-risk HPV-16 and HPV-18 E7 and low-risk HPV-11 E7, together with a plasmid expressing E6AP. Intriguingly, we observed that HPV-11 E7, like HPV-16 and HPV-18 E7, is stabilised by E6AP, as shown in Figure 33B. These results suggest that the presence of E6AP can stabilise the E7 protein from both low-risk and high-risk HPV types.

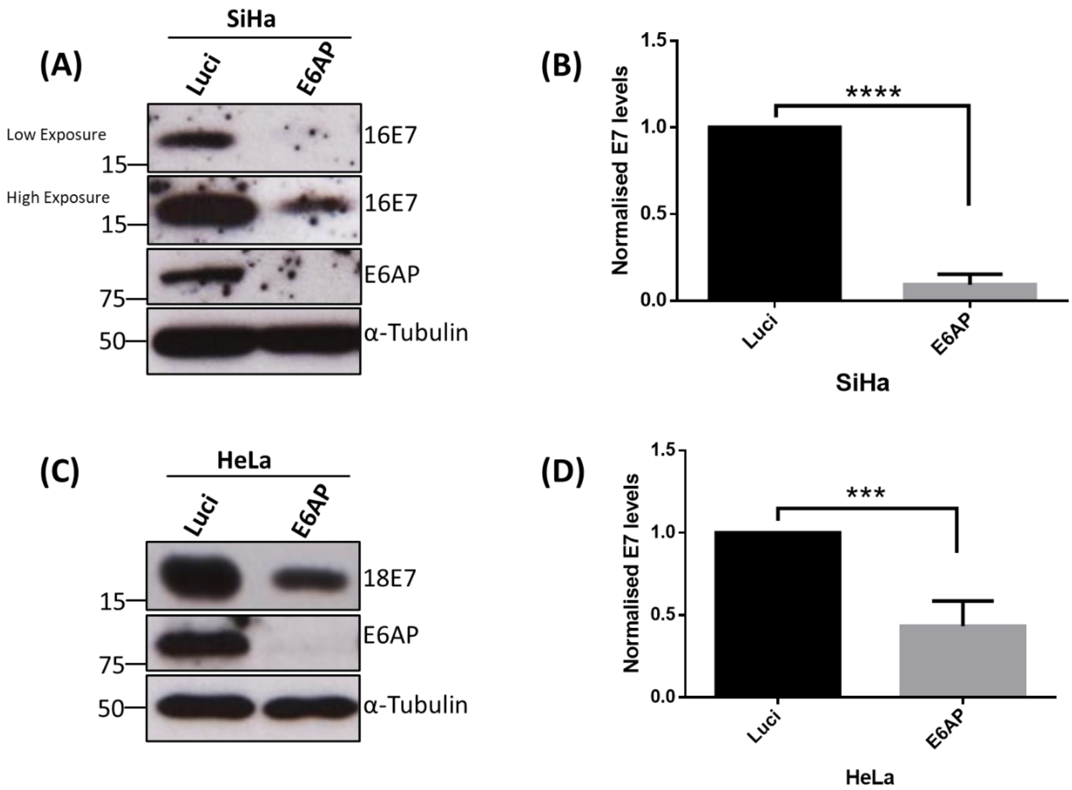


Figure 32. Loss of E6AP in HPV-positive cells leads to a decrease in E7 protein levels: SiHa **(A)** and HeLa **(C)** cells were transfected with siRNAs specific to E6AP and Luci (Control) followed by 72 hours' incubation. Cells were then harvested, and protein expression analysed by western blotting, using antibodies specific to HPV-16 E7, HPV-18 E7 and E6AP, α-tubulin was used as the loading control. **(B & D)** The statistical analysis of the E7 levels in the absence of E6AP, from at least three independent experiments, **** and *** represent p-values<0.001, statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

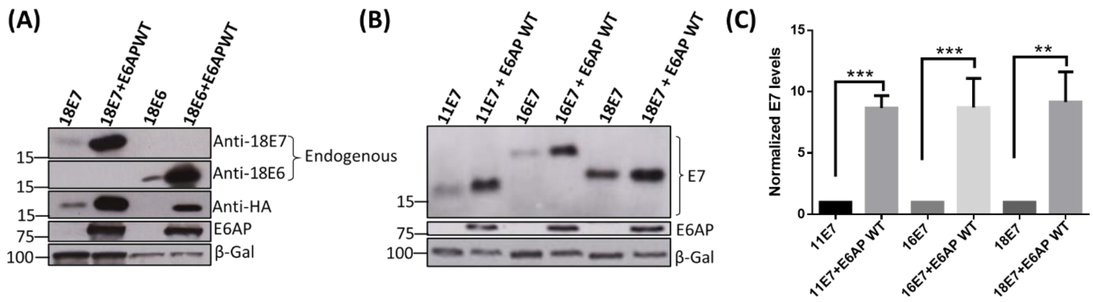
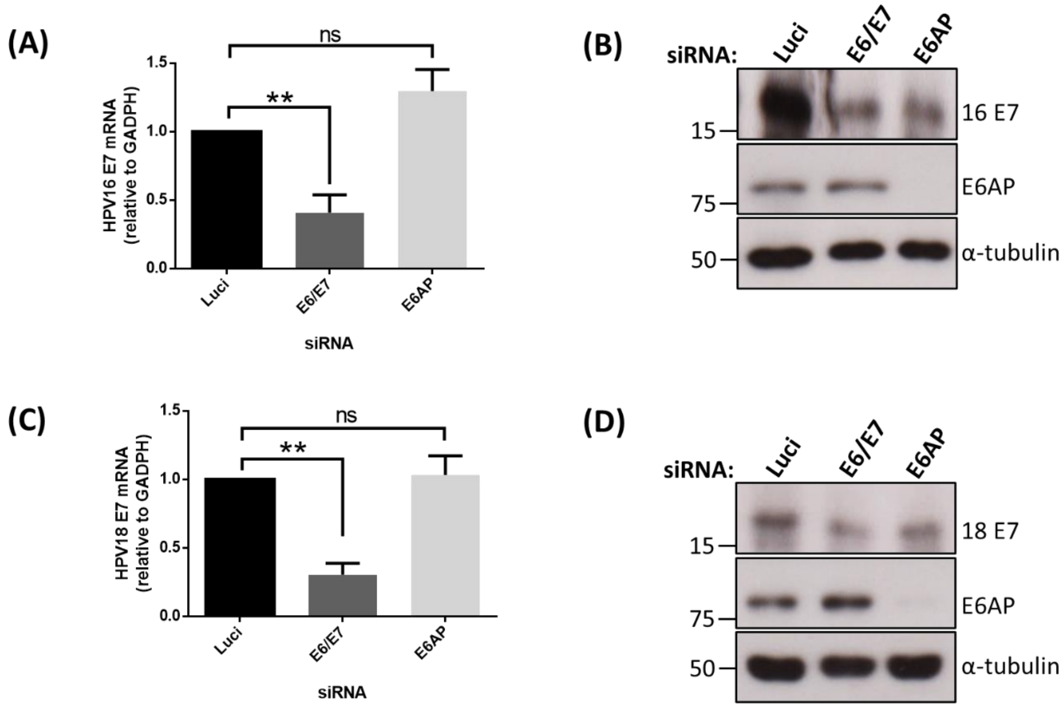


Figure 33. Ectopic expression of E6AP in HEK293 E6AP knockout cells increases E7 levels: HEK293 E6AP knockout cells were transfected with plasmids expressing HA-tagged HPV-11, HPV-16 and HPV-18E7 (100ng each) in the presence or absence of myc-tagged E6AP (1µg). **(A)** Represents the western blot analysis of the protein levels of HA-tagged 18E6 and 18E7 in the presence and absence of E6AP, probed using endogenous antibodies specific to 18E6 and 18E7, together with HA antibody, myc and β-gal. **(B)** Represents the western blot analysis for HPV-11, -16 and -18 E7 levels, using antibodies specific to HA, myc and β-gal (transfection loading control). **(C)** Represents the statistical analysis of the HA-tagged E7s normalised to β-gal using Student's t-test from at least 3 independent experiments. *** represents p-values <0.001 and ** represents p-values <0.005.

Knockdown of E6AP does not affect E7 RNA transcription

Tomaić *et al.*, in 2009, showed that ablation of E6AP expression leads to a decrease of E6 at the protein level, but does not cause any significant changes at the transcriptional level (Tomaic, Pim et al. 2009). To examine whether this also holds true in the case of E7, we transfected SiHa and HeLa cells with siRNA Luci (control), siRNA E6AP and siRNA E6/E7. After 72 hours, the cells were harvested, and total RNA was extracted and subjected to reverse transcription. The cDNAs were then amplified using the primers specific to HPV-16 E7 and HPV-18 E7, and the expression of E7 at the RNA level was assessed, as shown in Figure 34 A&C. It can be seen that the ablation of E6AP expression has no significant effect on either HPV-16 or HPV-18 E7 gene expression, when compared with that of the control-treated cells (Luci). However, the direct knockdown of E6/E7 resulted in a significant decrease in HPV-16 and HPV-18 E7 gene expression as expected, further validating the efficiency of the assay. Simultaneously, we also verified the protein levels of HPV-16 E7 (Figure 34B) and HPV-18 E7 (Figure 34D) by immunoblotting. Thus, these results demonstrate that silencing E6AP does not change E7 expression at the mRNA level.



Courtesy- Oscar Trejo

Figure 34. E7 RNA transcripts are not affected by E6AP silencing: SiHa **(A)** and HeLa **(C)** cells were transfected with siRNA specific to Luci (Control), E6AP and E6/E7. After 72 h, cells were harvested, and total RNA was extracted and reverse transcribed to generate complementary DNA (cDNA), which was diluted and subjected to real-time PCR amplification of the E7 transcript. Mean fold change of E7 mRNA of siRNA E6AP and siRNA E6/E7 was compared with E7 mRNA of siRNA Luci (control) cells were calculated and plotted. **(B&D)** Represents the corresponding western blot analysis validating the protein expression levels of 16 E7, 18E7, E6AP and the loading control α-tubulin.

In the absence of E6AP, E7 levels decrease in a proteasome-dependent manner

Several studies have shown that inhibiting the expression of E6AP leads to the degradation of E6 through the proteasome degradation machinery (Dukic, Lulic et al. 2020). Therefore, we wanted to examine whether E7 oncoprotein in the absence of E6AP also undergoes proteasome-mediated degradation. To do this, we repeated the knockdown of E6AP as described earlier in SiHa and HeLa cells. After 72 hours, cells were treated with proteasome inhibitor CBZ and DMSO as the control, followed by further incubation of 5 hours. Cells were then harvested, and E7 protein levels were analysed using western blotting. As can be seen in Figure 35, treatment with proteasome inhibitor leads to a dramatic increase in both HPV-16 E7 (Figure 35A) and HPV-18 E7 (Figure 35C) protein levels in the absence of E6AP. This suggests that when E6AP is absent, E7 undergoes degradation in a proteasome-dependent manner.

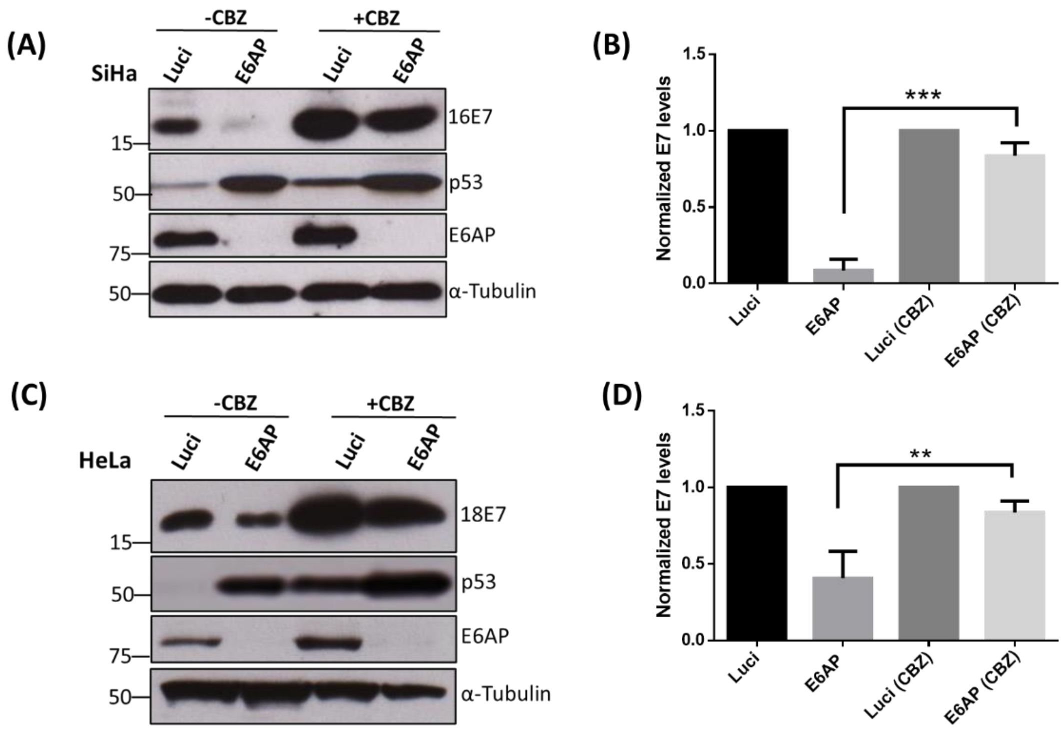


Figure 35. Blocking the proteasome degradation pathway rescues E7 protein levels in the absence of E6AP in HeLa and SiHa cells: siRNA mediated knockdown of E6AP was performed in SiHa **(A)** and HeLa **(C)** cells, together with control siRNA Luci. After 72 hours, cells were treated with proteasome inhibitor CBZ or DMSO (control) for 5 hours and then harvested, followed by western blot analysis using

antibodies specific for 16E7, 18E7, p53 and the loading control α-tubulin. **(B & D)** Statistical analysis of the increase in the E7 protein levels upon treatment with proteasome inhibitor CBZ, normalised to α-tubulin, from 3 independent experiments, performed using Student's t-test. *** and ** represent p-values <0.001 and 0.005, respectively.

The half-life of E7 decreases in the absence of E6AP

Having shown that the absence of E6AP leads to the degradation of E7 oncoprotein in a proteasome-dependent manner, we next wanted to determine whether this resulted from the increased turnover of HPV-16 and HPV-18 E7. To do this, we performed a half-life experiment in SiHa and HeLa cells in the presence and absence of E6AP, using siRNA against E6AP and siRNA luciferase as a negative control. After 72 hours, cells were then treated with cycloheximide to block further protein synthesis. The cells were harvested at 0, 15, 30, 45, 60 and 90 minutes post-treatment, followed by western blot analysis of HPV-16 and HPV-18 E7 protein levels. As can be seen in Figure 36, the half-life of E7 in cells treated with control siRNA Luci lies between 60 and 75 minutes for HPV-16 E7 and between 45 and 60 minutes for HPV-18 E7, as reported previously (Reinstein, Scheffner et al. 2000, Wang, Sampath et al. 2001, Basukala, Sarabia-Vega et al. 2020). However, in the cells treated with siRNA E6AP, the half-life of both HPV-16 and HPV-18 E7 is reduced by half and lies between 15 and 30 minutes. These results suggest that silencing E6AP in SiHa and HeLa cells leads to a dramatic increase the turn-over of HPV-16 and HPV-18 E7, similar to what is seen with the HPV E6 oncoprotein.

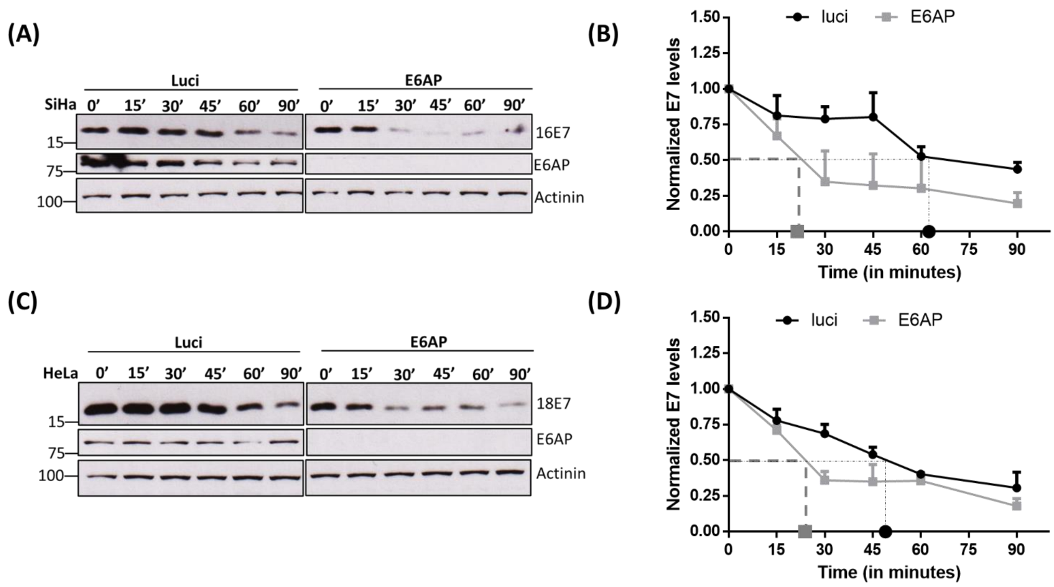


Figure 36. Knockdown of E6AP reduces the half-life of E7 oncoprotein in SiHa and HeLa cells: Expression of E6AP was silenced using siRNA E6AP, and siRNA Luci was used as control, in SiHa **(A)** and HeLa **(C)** cells. After 72 hours, cells were treated with cycloheximide for different time points between 0 and 90 minutes, after which the cells were harvested and E7 protein expression patterns were detected by immunoblotting using antibodies specific to HPV-16 E7, HPV-18 E7 and anti-E6AP antibody for E6AP; Actinin was used as the loading control. **(B&D)** Represents the graphs indicating the half-life of both 16 and 18E7 in the absence and presence of E6AP. Statistical analysis was performed using Student's t-test from 3 independent experiments, error bars represent the standard deviation. Dashed lines indicate the half-life of E7 in the absence of E6AP (grey line), while dotted lines indicate the half-life of E7 in the presence of E6AP (black line). The values were normalised with actinin for each time point.

In order to determine whether there was a specific pool of E7 that was being stabilised by E6AP we performed a series of immunofluorescence assays. As can been seen in Figure 37A, the ectopic expression of E6AP with HPV-16 and HPV-18 E7 in HEK293 E6APK/O cells stabilises both cytoplasmic and nuclear pools of E7, compared with the same cells expressing E7 in the absence of E6AP. In addition, when we blocked the proteasome degradation machinery by treating the HPV-16 and HPV-18 E7-expressing cells with CBZ (as shown in Figure 37B), this led to the dramatic rescue of both nuclear and cytoplasmic pools of E7. Taken together, these data suggest that E6AP stabilises and protects E7 from proteasome degradation in the nucleus as well as in the cytoplasm of the cells.

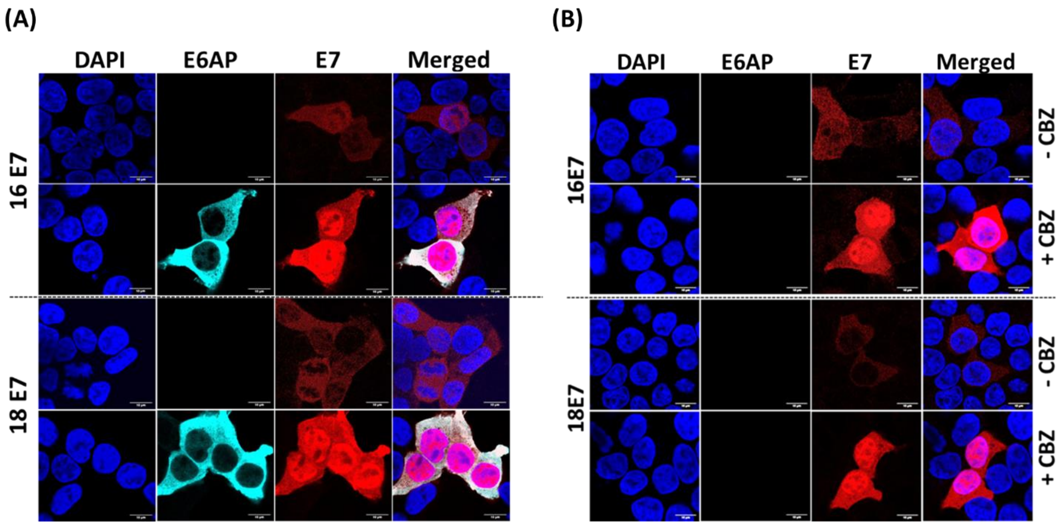


Figure 37. Both nuclear and cytoplasmic pools of E7 oncoprotein are stabilised in the presence of E6AP and proteasome inhibitor CBZ: (A) Plasmids expressing Flag/HA-tagged HPV-16 E7 (100ng) and HPV-18 E7 (100ng), together with myc-tagged wildtype E6AP (1µg), were co-transfected in HEK293 E6APK/O cells. (B) HEK293 E6AP K/O cells were transfected with Flag/HA-tagged HPV-16 E7 (100ng) and HPV-18 E7 (100ng). After 24 hours, cells were treated with proteasome inhibitor CBZ and DMSO as the control for 5 hours. Cells from A&B were then fixed, and immunostained for E7 and E6AP proteins using antibodies for HA and E6AP. The nuclei of the cells were stained with DAPI. The scale bar represents 10µm.

The absence of E6AP leads to E7 Ubiquitination

We next wanted to determine whether the absence of E6AP could lead to the ubiquitination of the E7 oncoprotein. To investigate this, we co-transfected HEK293 E6AP K/O cells with plasmids expressing Flag/HA-tagged HPV-16 or HPV-18 E7, myc-tagged E6AP WT and HA-tagged Ubiquitin. After 24 hours, cells were harvested and immunoprecipitated using anti-Flag-conjugated agarose beads. The ubiquitinated HPV-16 E7 (Figure 38A) and HPV-18 E7 (Figure 38B) were then detected by immunoblotting using an anti-HA-HRP conjugated antibody. Interestingly, we observed a marked increase in the polyubiquitination of both HPV-16 and HPV-18 E7 in the absence of E6AP. This result suggests that the presence of E6AP protects HPV-16 and HPV-18 E7 from undergoing polyubiquitination.

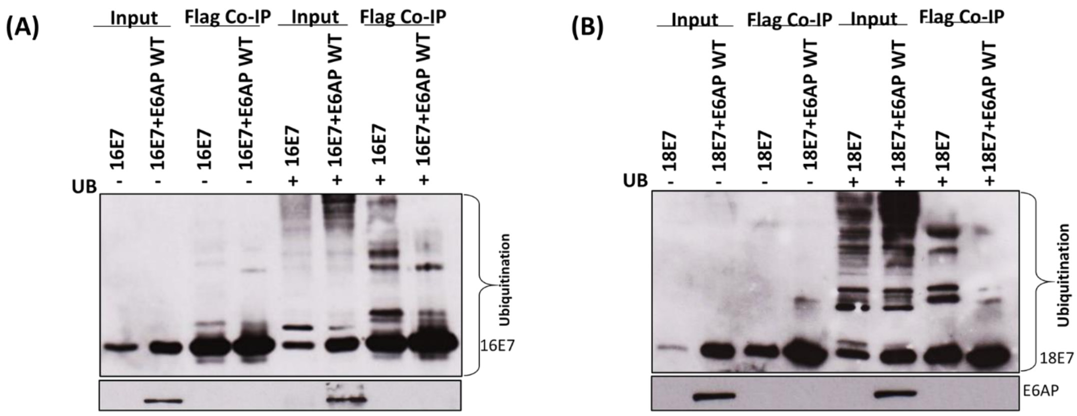
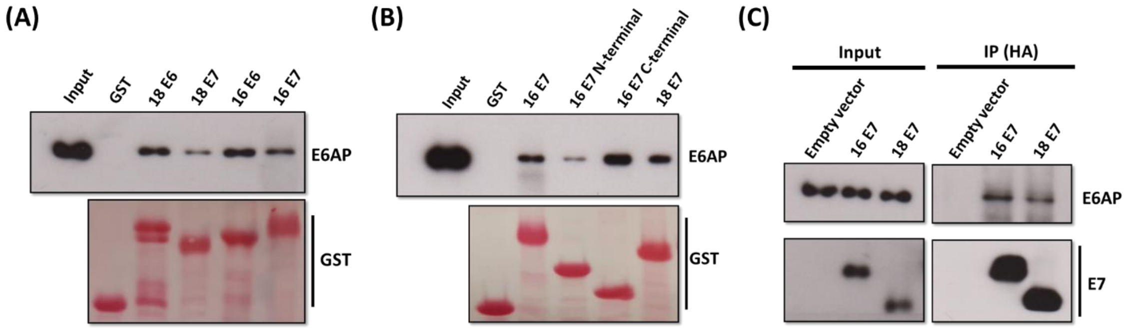


Figure 38. HPV-16 and HPV-18 E7 undergo ubiquitination in the absence of E6AP: HEK293 cells were co-transfected with plasmids expressing FLAG/HA-tagged HPV-16 E7 **(A)** and HPV-18 E7 **(B)**, HA-tagged Ub, and myc-tagged E6AP. After 24 hours, cells were harvested and subjected to immunoprecipitation with anti-FLAG-conjugated agarose beads. Polyubiquitinated E7 was detected using immunoblotting with anti-HA-HRP antibody and E6AP using anti-myc antibody.

E6AP interacts with HPV E7

Having shown that E6AP can directly stabilise HPV E7, were interested in determining if E7 could interact physically with E6AP. To investigate this, we first performed a GST pull-down assay using GST-tagged HPV-18 E6 and E7, GST-tagged HPV-16 E6 and E7, and GST alone as a negative control. HEK293 E6APK/O cells were transfected with plasmid expressing myc-tagged E6AP. After 48 hours, cells were harvested, and pull-down assays were performed using the GST-tagged proteins, as indicated in Figure 39A. As expected, we observed that both HPV-16 and HPV-18 E6 bind to E6AP, in agreement with previous studies (Scheffner, Werness et al. 1990, Huibregtse, Scheffner et al. 1993). In addition, we observed that both HPV-16 and HPV-18 E7 interact with E6AP, but noticeably less strongly than the HPV-E6. We next wanted to ascertain which region, whether N-terminal or C-Terminal, of E7 binds to E6AP. To examine this, we repeated the GST-pull down assay, using GST-tagged HPV-16 E7 together with GST- tagged HPV-16 E7 N-terminus, GST- tagged HPV-16 E7 C-terminus and GST- tagged HPV-18 E7, plus GST alone as the negative control. The results in Figure 39B clearly show that full-length HPV-16 and HPV- 18 E7 bind to E6AP; most significantly, however, it is clear that the C-terminal region of HPV-16 E7 binds more strongly to E6AP than the N-terminal region. We further validated these results using a co-immunoprecipitation assay in HEK293 E6APK/O cells co-transfected with plasmids expressing HA/FLAG-tagged HPV-16 or HPV-18 E7, together with myc-tagged E6AP, or an empty vector as a negative control. After 48 hours, cells extracts were isolated and co-immunoprecipitated using anti-HA immobilised on agarose beads. It is clear from the results shown in Figure 39C, that E6AP binds to both HPV-16 and HPV-18 E7, validating the results obtained from the GST pull-down assays. Taken together, these results demonstrate that HPV E7 oncoprotein, like HPV E6 oncoprotein, can interact with the E6AP ubiquitin ligase.



Courtesy- Oscar Trejo

Figure 39. E7 oncoprotein binds E6AP: (A & B) GST pull-down assays were performed using the cell lysates ectopically expressing myc-tagged E6AP in HEK293 E6AP knockout cells, together with purified GST proteins, as indicated. The top panels show the immunoblot analysis for E6AP protein probed using anti-myc antibody, and the lower panels show the Ponceau stain for different GST fusion proteins. **(C)** Cell lysates from HEK293 E6AP knockout cells expressing Myc-tagged E6AP, Flag/HA-tagged 16E7, 18E7 and pCDNA3 empty vector (as control) were co-immunoprecipitated with anti-HA-conjugated agarose beads, followed by analysis by western blotting. E6AP protein was probed using anti-myc antibody and E7 was probed using anti-HA HRP conjugated antibody.

E6AP mediated stabilisation of E7 increases the degradation of pRB family of proteins

Previous studies have shown that the high-risk HPV E7 oncoprotein targets the pRb tumor suppressor and related pocket protein family members, p107 and p130, for degradation, in turn driving cell cycle progression (Smotkin and Wettstein 1986, Morris and Dyson 2001). We, therefore, next were interested in ascertaining whether E6AP-directed stabilisation of high-risk HPV E7 oncoprotein could lead to increased degradation of E7's cellular targets, pRb, p107 and p130. To investigate this, we decided to perform *in vivo* degradation assays in HEK293 E6APK/O cells co-transfected with plasmids expressing HA-tagged pRB (Figure 40A), p107 (Figure 40B) and p130 (Figure 40C) together with HA/FLAG-tagged HPV-16 E7 and myc-tagged E6AP. After 24 hours, we harvested the cells and performed immunoblotting to examine any changes in the protein levels of pRb, p107 and p130 induced by E7 in the presence and absence of E6AP. As can be seen in Figure 41, both pRB, p130 and p107 protein levels show a modest decrease in the absence of E6AP. More interestingly, however, in the presence of E6AP, we observed that pRB, p130 and p107 protein levels all showed a dramatic decrease, corresponding with a marked stabilisation in E7 levels. These results suggest that increased stabilisation of E7 by E6AP generates a functionally active E7 protein.

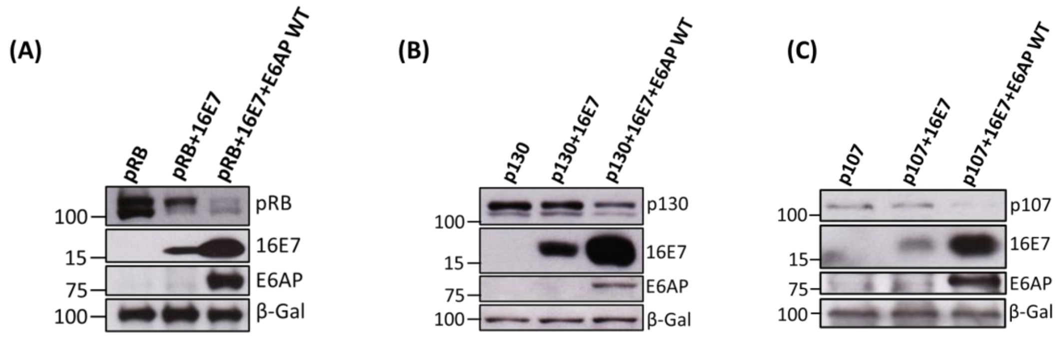


Figure 40. E6AP-stabilised E7 increases the degradation of pRB, p130 and p107: HEK293 E6AP knockout cells were transfected with plasmids expressing HA-tagged pRb (2.5µg) **(A)**, HA-tagged p130 (2µg) **(B)** and HA-tagged p107 (2µg) **(C)**, together with FLAG/HA-tagged 16E7 (100ng) and myc-tagged E6AP (1µg). After 24 hours, cells were harvested, and protein expression was analysed by western blotting using anti-HA HRP-conjugated antibody and anti-myc antibody. β-gal was used as loading and transfection efficiency control.

E6 oncoprotein regulates the stabilisation of E7 oncoprotein by E6AP

The above studies raise the intriguing prospect that there may be potential regulation of E6 and E7 protein levels in manner which is interdependent and centred around E6AP. To investigate this possibility, we co-transfected HEK293 E6APK/O cells with plasmids co-expressing HA/FLAG-tagged HPV-16 E7 and HA-tagged HPV-16 E6, together with myc-tagged E6AP. After 24 hours, cells were harvested, and the effects of E6 on E6AP-mediated E7 stabilisation were examined by western blot. The results shown in Figure 41 demonstrate, as expected, that E7 and E6, when expressed individually with E6AP, are significantly stabilised. However, surprisingly, we observed that E7 protein levels are reduced considerably in the presence of E6AP when expressed together with E6, suggesting that E6 may regulate the E6AP-directed stabilisation of E7 oncoprotein.

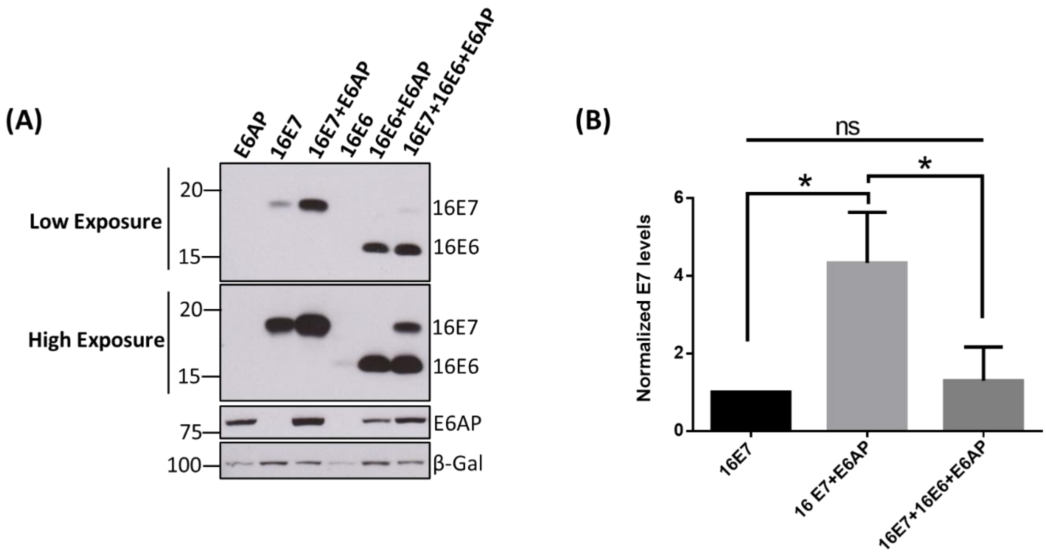


Figure 41. E6 oncoprotein regulates the E6AP-mediated stabilization of E7 oncoprotein: (A) HEK293 E6AP knockout cells were transfected with the plasmids expressing HA-tagged HPV-16 E6 (3µg) and HA/FLAG-tagged HPV 16 E7 (100ng), together with myc-tagged E6AP (1µg). Cells were harvested after 24 hours and subjected to immunoblot analysis using anti-HA HRP-conjugated antibody and anti-myc antibody. β-gal was used as loading and transfection efficiency control. **(B)** Represents the changes in protein expression patterns of HPV E7 induced by E6AP in the absence and presence of HPV E6. Statistical analysis was performed using Student's t-test from 3 independent experiments; error bars represent the standard deviation. The values were normalised with transfection loading control, β-gal.

Section III

E6 is phosphorylated in the absence of E6AP

Previous studies have shown that HPV E6 oncoproteins are phospho-regulated by several cellular kinases either directly or indirectly (Boon, Tomaic et al. 2015, Thatte, Massimi et al. 2018). E6 is very weakly phosphorylated during the normal cell cycle, but this increases drastically upon the induction of DDR pathways in cervical cancer-derived cell lines (Thatte, Massimi et al. 2018). Since loss of E6AP induces a potent stress response, involving upregulation of p53, induction of apoptosis and loss of E6 protein (Vande Pol and Klingelhutz 2013), we were interested in investigating how this loss of E6AP might impact upon signalling to the E6 oncoprotein. To examine this, we decided to knock down the expression of E6AP and E6/E7 in cervical cancer-derived HeLa cells, using siRNAs targeting E6AP and the E6/E7 oncoproteins. After 72 hours, cells were harvested, and the extracts were analysed by western blotting, using antibodies raised against HPV-18 E6 to detect both phosphorylated and non-phosphorylated forms. As can be seen in Figure 42, knockdown of E6AP and E6/E7 leads to a reduction in the levels of total E6, in agreement with previous studies (Tomaic, Pim et al. 2009). However, despite an overall reduction in E6 protein levels, we also observed a dramatic increase in the levels of the phosphorylated form of HPV-18 E6 (pE6) upon knockdown of E6AP. However, pE6 remains undetectable in the cells transfected with si E6/E7, similar to the si-Luciferase control (Luci).

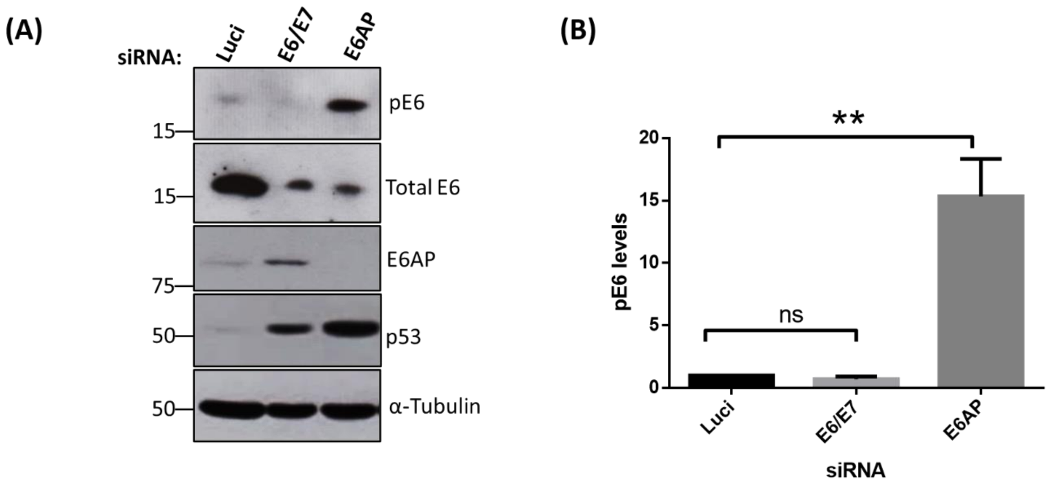


Figure 42. Phosphorylation of HPV 18E6 upon E6AP knockdown: (A) HeLa cells were subjected to siRNA-mediated knockdown of E6AP and E6/E7, with Luciferase (Luci) as control. After 72 hours, cells were harvested and analysed by western blot, using antibodies specific to total E6, phosphorylated E6 (pE6) and E6AP; α-tubulin was used as a loading control. (B) The histogram shows the statistical significance of changes in pE6 in the absence of E6AP, from at least three independent experiments; ** represents p value<0.005 and ns represents non-significant; statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

To ascertain what causes the phosphorylation of E6 upon silencing of E6AP, we performed another knockdown experiment in HeLa cells: this time, we knocked down E6AP together with p53, to see whether E6 is phosphorylated in response to p53 activation, since p53 is known to be the major degradation target of the E6/E6AP complex. We also included a double knockdown of siE6AP and siE6/E7 to verify the identity of the pE6 moiety. As can be seen in Figure 43, loss of E6AP induces a marked increase in the levels of pE6, and this is reduced in the presence of siE6/E7. Most interestingly, this phosphorylation of E6 in the absence of E6AP is dependent upon the presence of p53, since transfection of siRNA p53 abolishes the phosphorylation of E6. Taken together, these results demonstrate that p53 is required for phosphorylation of E6 in the absence of E6AP.

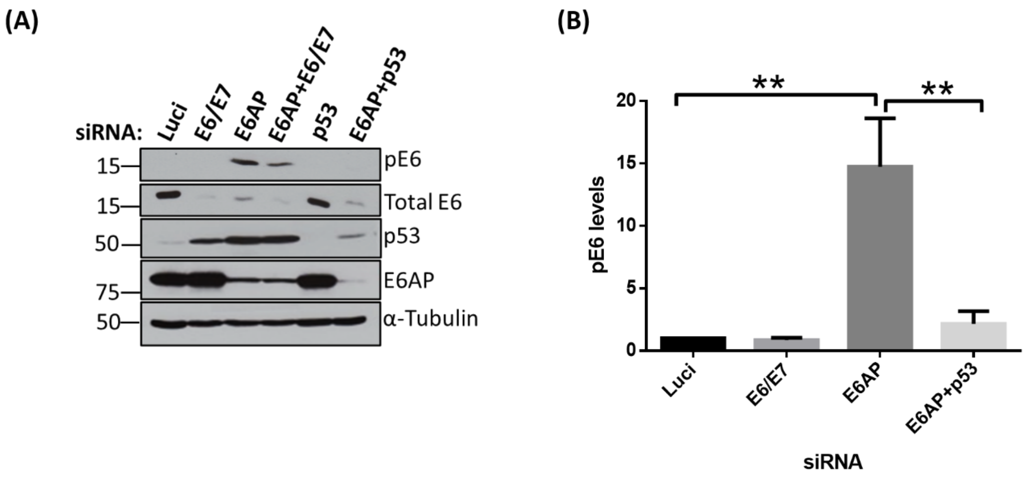


Figure 43. Knockdown of E6AP induces phosphorylation in response to p53 activation: (A) HeLa cells were transfected with siRNAs against E6AP, E6/E7 and p53. After 72 hours, cells were harvested and analysed by western blotting. Proteins were probed using antibodies specific to pE6, total E6, p53 and E6AP; α-tubulin was used as a loading control. (B) The histogram shows the statistical significance of changes in pE6 levels in the absence of E6AP and in the absence of E6AP + p53, from three independent experiments with the p-value of <0.005 (represented with **); statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

We further validated this in the p53-null H1299 cell line. For this, we first knocked down the E6AP and after 48 hours cells were transfected with p53, wild-type 18E6 and 18E6 delPBM expression constructs, followed by further incubation for 24 hours. Cells were then harvested and pE6 levels were analysed using western blotting. We observed that the levels of pE6 increase markedly in the absence of E6AP and in the presence of the p53, as shown in Figure 44. Taken together with the data shown in Figure 43, this suggests that E6, in the absence of E6AP, is phosphorylated in response to the upregulation of p53.

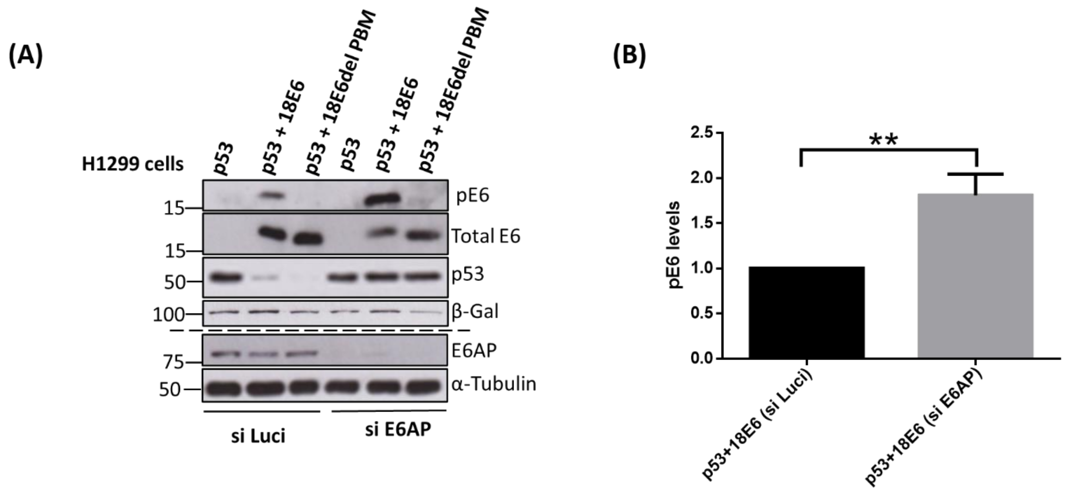


Figure 44: Knockdown of E6AP and over-expression of p53 in p53-null H1299 cells leads to increased E6 phosphorylation: (A) H1299 cells were transfected with si E6AP and si Luci (as control). After 48 hours, the same cells were transfected with plasmids expressing Flag-tagged p53 (500ng), HA-tagged 18E6 (wild-type) (5µg), and HA-tagged 18E6 delPBM (5µg), together with a LacZ expression plasmid (Transfection efficiency control) (100ng), followed by further incubation of 24 hours. Protein analysis was performed by western blot using specific antibodies against p53, p18E6 and HA (for probing 18E6). β-gal was used as a loading control for the over-expressed proteins, while α-tubulin was used as a loading control for E6AP siRNA knockdown. (B) The histogram represents the statistical significance of changes in pE6 in the absence of E6AP and presence of over-expressed p53 protein, from at least three independent experiments; ** represents p value<0.005 and ns represents non-significant; statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

DNA PK phosphorylates E6 in the absence of E6AP

Having found that E6 is phosphorylated in a p53-dependent manner following knockdown of E6AP, we were interested in identifying the kinase responsible. We had shown previously that various stress-response kinases can strongly phosphorylate E6, including Chk1 and Chk2 via PKA, and another, as yet unidentified, kinase activated by cycloheximide treatment (Thatte, Massimi et al. 2018). Therefore, we initially focussed our attention on CHK1 and PKA, repeating the siRNA E6AP transfection in HeLa cells, but also including CHK1 and PKA inhibitors. In parallel, as a control, we also induced DNA damage in HeLa cells by treating the cells with H_2O_2, in the presence of the PKA inhibitor, to verify the phospho-regulation of E6 by PKA. The results in Figure 45A and Figure 45B show that there are no changes in the levels of pE6 in the presence of either the CHK1 or PKA inhibitors in the absence of E6AP. However, the inhibition of PKA drastically reduces the levels of pE6 in H_2O_2-treated cells (Figure 45C), in agreement with the previous study (Thatte, Massimi et al. 2018). Taken together, these results suggest that neither PKA nor CHK1 kinase is involved in the phosphorylation of pE6 when E6AP is knocked down.

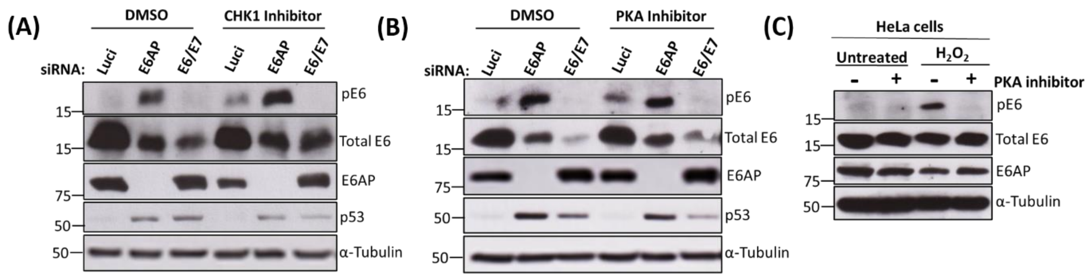


Figure 45. Neither CHK1 nor PKA phosphorylates E6 in the absence of E6AP: HeLa cells were transfected with siRNAs against E6AP, E6/E7, and luciferase (Luci), as control. After 60 hours, CHK1 (100nM) (A) and PKA (15µM) (B) inhibitors, and DMSO as control, were added to the cells, followed by further incubation for 12 hours. Cells were then harvested, and samples were analysed using western blotting with the antibodies against total E6, pE6 and E6AP; α-tubulin was used as a loading control. (C) HeLa cells were treated with PKA inhibitor (15µM) for 15 hours, followed by treatment with DMSO (as control) or H_2O_2 (500µM) for 4 hours. Western blots were probed using antibodies specific against total E6, pE6 and α-tubulin (loading control).

In order to search for other potential kinases, we performed an *in-silico* kinase analysis using Netphos 3.1 server software and identified DNA PK as a high-confidence candidate, as shown in Figure 46A. To test this, we performed an *in vitro* kinase assay using purified DNA PK with purified HPV-18 and HPV-16 E6 glutathione-S-transferase (GST) fusion proteins in the presence of radioactively-labelled ATP. The results shown in Figure 46B demonstrate that both HPV-18 and HPV-16 E6 are excellent substrates for phosphorylation by DNA PK *in vitro*. To confirm that the phosphorylation was on the T156 residue, the assay was repeated using a panel of E6 mutants. The results in Figure 46C demonstrate that any modification to the E6 PBM reduces phosphorylation by DNA PK, and that the phospho-acceptor site is T156.

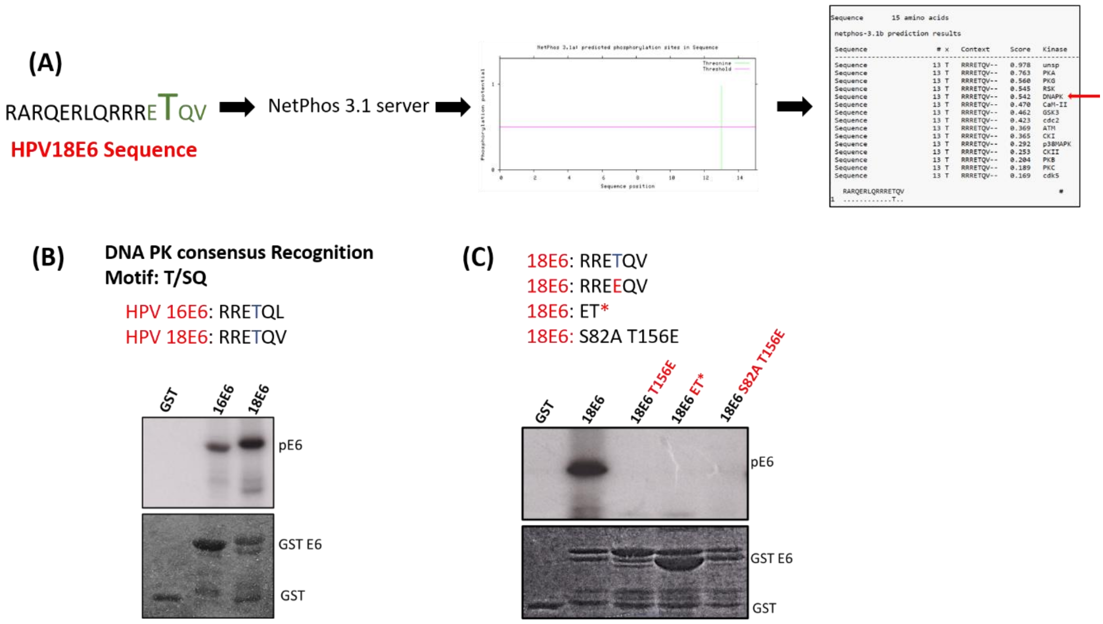


Figure 46. DNA PK phosphorylates HPV 18-E6 in vitro*:* (A) Represents *in silico* kinase analysis using NET Phos3.0 software for the HPV 18E6 PBM, specifically the T156 residue. (B) *In vitro* phosphorylation assay using purified GST fusion proteins for wildtype HPV-16, and HPV-18 E6, and HPV-18 E6 mutated within its PBM (C) incubated with purified DNA PK enzyme, in the presence of [ϒ-^{32}P] ATP. (Top) Autoradiograms. (Bottom) Coomassie-stained SDS-PAGE gels, indicating the pE6, the GST fusion E6 protein, and GST control.

To ascertain whether DNA PK is involved in E6 phosphorylation in vivo, HeLa cells were transfected with siRNA E6AP, followed by treatment with DNA PK inhibitor for 12 hours. The cells were harvested and analysed by western blotting. The results in Figure 47 demonstrate that the levels of pE6 are greatly decreased in the presence of the DNA PK inhibitor, confirming DNA PK as the kinase responsible for phosphorylating E6 after removal of E6AP.

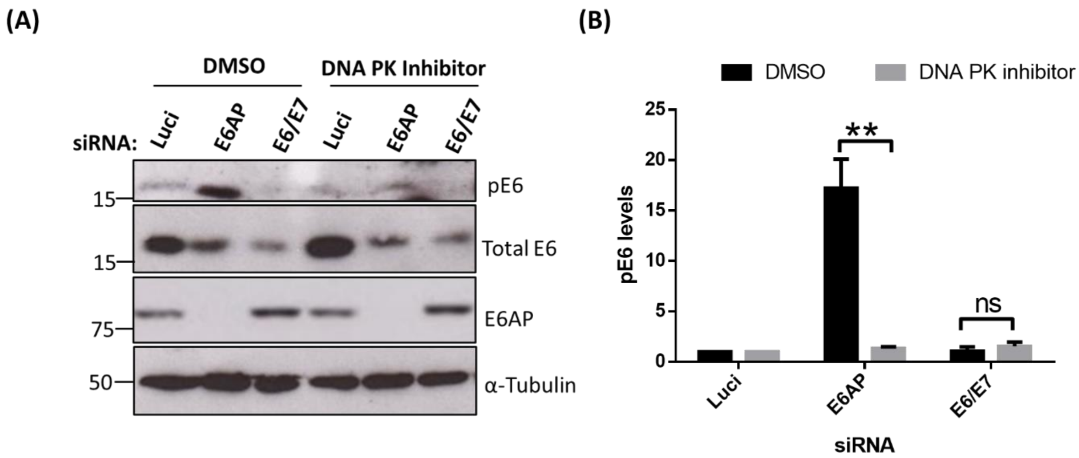


Figure 47. DNA PK phosphorylates HPV 18-E6 in vivo in the absence of E6AP: (A) HeLa cells were transfected with siRNAs against E6AP, E6/E7 and Luciferase, as the control. After 60 hours, DNA PK inhibitor (10µM), and DMSO as control, were added to the cells, which were incubated for a further 12 hours. Cell extracts were then analysed by western blot using antibodies for total E6 and pE6; α-tubulin was used as a loading control. (C) Represents the statistical significance of the pE6 levels from at least three independent experiments in the presence of DNA PK inhibitor with a p-value of <0.005 (represented with **); statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

Although all high-risk HPV E6s have a conserved PBM at their carboxy-terminus, there is a high degree of variation in their sequences upstream and downstream of the phospho-acceptor site, as shown in Figure 48A. Having shown that T156, within the HPV-18 E6 PBM, plays a critical role in kinase recognition, we wanted to ascertain whether this is conserved between E6 proteins from different HPV types. To investigate this, we performed a series of *in vitro* kinase assays using purified DNA PK with different HPV E6 GST-fusion proteins, in the presence of radiolabelled ATP. As can be seen in Figure 48B, E6 proteins from HPV-16, HPV-18, HPV-39 and HPV-68 are heavily phosphorylated by DNA PK; those of HPV-33 (potentially due to the presence of Alanine at position -1), HPV-51 and HPV-58 are mildly phosphorylated; and E6s from HPV-35 (perhaps because of the presence of Glutamic acid downstream of Threonine at position -1) and HPV-56 are only weakly phosphorylated. These results suggest that, in agreement with previous studies, the non-canonical residues play critical roles in kinase recognition.

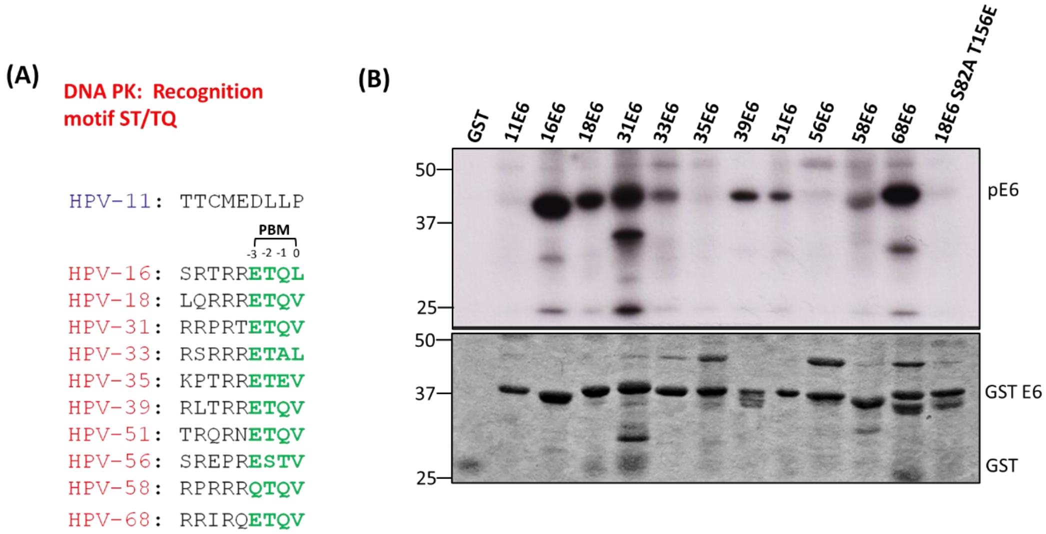


Figure 48. Different HPV E6 types display different levels of phosphorylation: (A) The carboxy-terminus sequence of E6 proteins from different HPV types. (B) The purified GST fusion proteins were incubated with DNA PK enzyme and [γ-32P] ATP. The phosphorylation levels of the E6 proteins were then analysed by SDS-PAGE and autoradiography. The upper panel shows the autoradiogram of different *in vitro* phosphorylated 18E6 GST fusion proteins; the lower panel shows the Coomassie blue-stained gel.

Having shown that DNA PK can phosphorylate E6 in vivo following the loss of E6AP, we were interested in determining whether this was specific to loss of E6AP, or whether other DNA damage-induced stress responses could trigger DNA PK phosphorylation of E6. To do this, HeLa cells were first treated with the DNA PK inhibitor, followed by treatment with Teniposide, which we have shown previously can induce a high level of E6 phosphorylation (Thatte, Massimi et al. 2018). As can be seen from Figure 49, the DNA PK inhibitor causes a marked decrease in the levels of E6 phosphorylation in response to loss of E6AP, however DNA PK inhibition has no effect on the levels of pE6 following treatment with Teniposide. These results demonstrate that DNA PK activation is a specific response to the loss of E6AP.

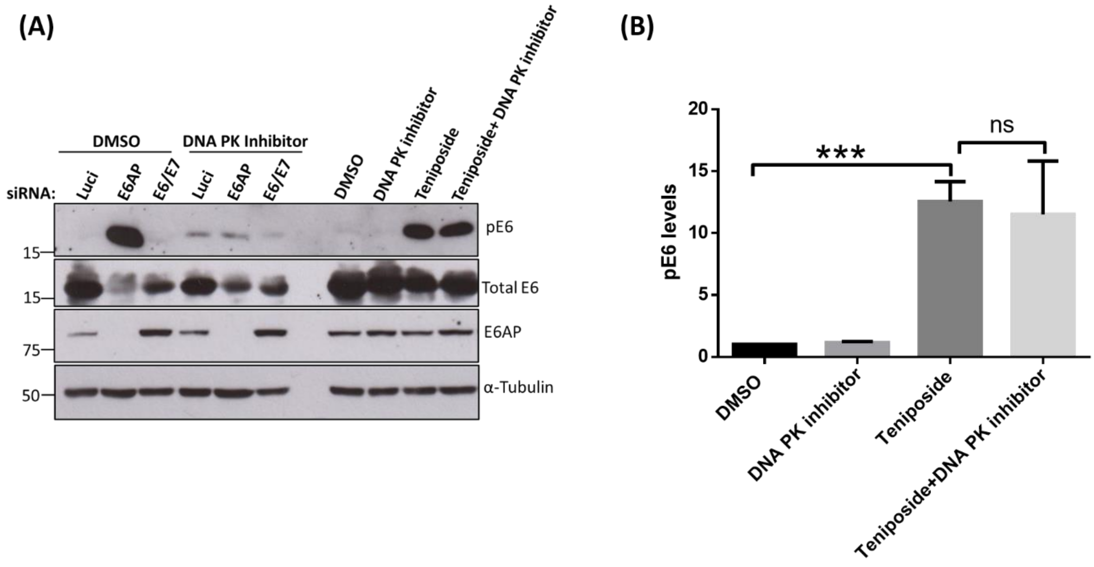


Figure 49. DNA PK does not phosphorylate E6 upon exogenous induction of DNA damage: (A) In the first set, HeLa cells were transfected with si E6AP, si E6/E7 and si Luci as control. After 48 hours, we added DNA PK inhibitor (10µM), or DMSO, as control. A second set of cells was also treated with the DNA PK inhibitor (10µM), or DMSO, as control. Both sets of cells were then incubated for 12-15 hours, then treated with a DNA Damaging agent; Teniposide (at 5 µM), followed by further incubation for 4 hours. Cells were then harvested and analysis was performed by western blot using specific antibodies against pE6, Total E6 and E6AP, plus α-tubulin as loading control. (B) The histogram represents the statistical significance of changes in pE6 levels upon treating the cells with Teniposide in the presence and absence of DNA PK inhibitor. The results from three independent experiments are shown, with ***p-value <0.005; ns represents non-significant. Quantification was performed using Student's t-test; error bars indicate the standard deviation of the mean.

Transcriptional activity of p53 regulates the phosphorylation of E6 in the absence of E6AP

Having found that phosphorylation of E6 was dependent upon p53 and DNA PK, we next wanted to determine whether p53 transcriptional activity was required. To examine this, we repeated the knockdown of E6AP in HeLa cells, and then treated the cells with α-pifithrin (Leker, Aharonowiz et al. 2004, Li, Ghiani et al. 2008), to block p53 transcriptional activity. After a further 48hrs, the cells were harvested and pE6 levels ascertained by western blotting. The results in Figure 50 show that the levels of pE6 decrease significantly in the presence of α-pifithrin in comparison with control-treated cells. These results demonstrate that loss of E6AP results in phosphorylation of HPV-18 E6, in a manner that requires both p53 transcriptional activity and DNA PK.

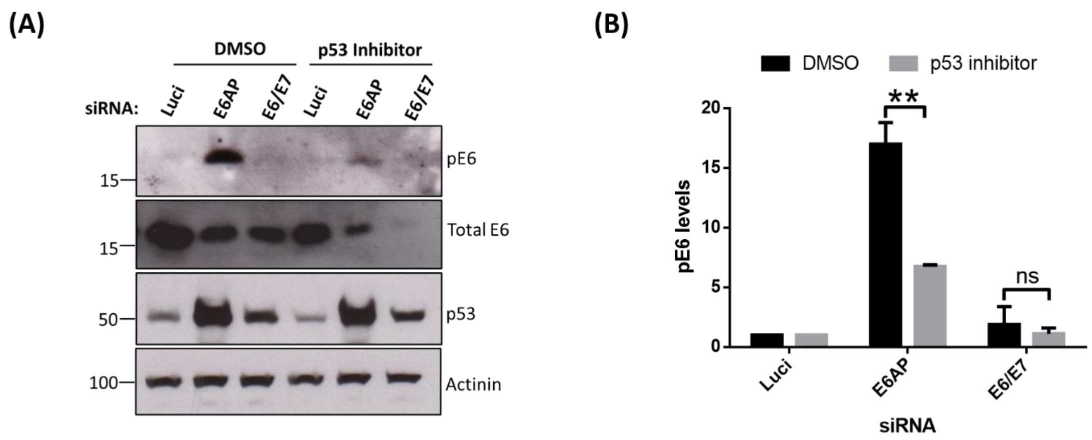


Figure 50. p53 transcriptional activity controls the phosphorylation of E6 upon E6AP knockdown:(A) siRNAs targeting E6AP, E6/E7 and Luciferase control were transfected into HeLa cells, followed by incubation for 24 hours, after which p53 inhibitor α-pifithrin (30µM), or DMSO as control, were added to the cells. Cells were then further incubated for 48 hours, after which they were harvested and analysed by western blotting using antibodies specific to pE6, and total E6; α-tubulin was used as a control. (B) The histogram shows relative statistical significance of changes in pE6 levels in the presence of p53 inhibitor and absence of E6AP from at least three independent experiments with p-value of <0.05 (represented with *), ns represents non-significant.

The E6/E6AP-mediated degradation of p53 is considered to be an important mechanism in the initiation and development of cervical cancers. Several studies have shown that disrupting this complex, either by knocking down E6AP or by treating the cells with inhibitors that lead to the accumulation of p53, further induces apoptosis in cancer cells in a p53-dependent manner (Hietanen, Lain et al. 2000, Beaudenon and Huibregtse 2008, Lee, Kwon et al. 2010, Shai, Pitot et al. 2010, Zhao, Szekely et al. 2010). It was possible that this imbalance between cell proliferation and apoptosis upon the activation of p53 in the absence of E6AP might induce cellular stress, in turn activating the stress-responsive kinases, further leading to the phosphorylation of E6. To investigate this, we repeated the knockdown experiment in HeLa cells, as described above, and checked for the levels of stress marker ^{pS139} γH2AX, which is known to increase upon activation of the DNA damage-signalling pathway and also upon induction of the apoptotic signalling cascade (Rogakou, Nieves-Neira et al. 2000). Interestingly, upon analysis, we observed that the levels of ^{pS139} γH2AX increase dramatically in the E6AP-knockdown cells compared with the Luci control, and decrease significantly upon the knockdown of both E6AP and p53, as shown in Figure 51A. This suggests that restoring the activity of p53 after silencing E6AP can induce cellular stress that increases ^{pS139} γH2AX levels in HeLa cells. Next, we wanted to ascertain if it is regulated through the transcriptional activity of p53. To test this, we repeated the siRNA knockdown experiment in the presence and absence of α-pifithrin, an inhibitor of p53 transcriptional activity. Similar to the previous observation, knockdown of E6AP increases the levels of ^{pS139} γH2AX, which decreases greatly upon treating the cells with α-pifithrin (Figure 51B). This further suggests that, in the absence of E6AP, the transcriptional activity of p53 induces stress in the cells that may result in the activation of DNA PK, which eventually phosphorylates E6.

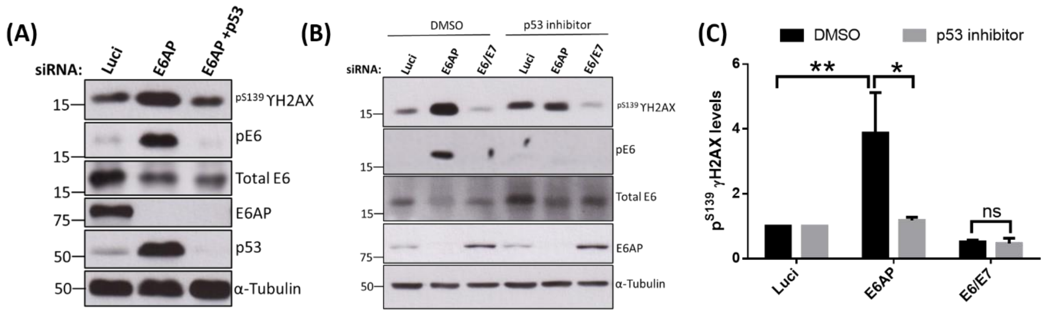


Figure 51. p53 transcriptional activity increases pS139γH2AX levels in the absence of E6AP: (A) siRNAs targeting E6AP and p53, plus si-luciferase (Luci) control were transfected into HeLa cells, and incubated for 72h. Cell were then harvested and analysed by western blotting. (B) HeLa cells were transfected with siRNA against E6AP and E6/E7, plus Luci as control. After 24 hours, cells were treated with p53 inhibitor α-pifithrin (30 µM) for a further 48 hours. Cells were then harvested and analysed by western blotting. Proteins were probed using specific antibodies; pS139γH2AX, E6AP, pE6 and total E6; α-tubulin was used a loading control. (C) The histogram represents the statistical significance of the increase in pS139γH2AX in the absence of E6AP and in response to p53 transcriptional activation from three independent experiments; * represents the p-value of <0.05 and ** represents the p-value of <0.005; statistically quantified using Student's t-test; error bars indicate the standard deviation of the mean.

pE6 in the absence of E6AP negatively regulates the transcriptional activation of p53-responsive genes

Thatte *et al*, in 2018, showed that phosphorylation of the E6 PBM plays a crucial role in blocking the transcriptional activity of p53-responsive genes. We wanted to determine whether the pool of E6 that is phosphorylated in the absence of E6AP could functionally block the transcriptional activity of p53. Therefore, we initiated a series of studies to examine whether the phosphorylation of the E6 PBM has any effect on p53 transcriptional transactivation of p21 and mdm2 promoters, using *Renilla* luciferase as a transfection efficiency control.

To examine this, we first transfected the cells with si E6AP or si Scramble (as control). After 48 hours, the cells were transfected with appropriate reporter constructs, together with plasmids expressing p53, wild-type 18E6 and 18E6 delPBM (phospho mutant). After a further 24 hours, the cells were harvested and luciferase activity was measured using the dual-luciferase system; the results are shown in Figure 52 (A&B) and the expression profiles of all the proteins was verified using the western blot as shown in figure 52 C. As expected, the transcriptional activity of p53 is inhibited by both wild type 18E6 and the 18E6 delPBM in the presence of E6AP, since p53 degradation is induced by the E6/E6AP complex. However, in the absence of E6AP, we observed that 18E6 delPBM fails to inhibit the transcriptional activation of p53-responsive genes, while the wildtype E6 retains the ability to inhibit p53 transcriptional activity for both p21 and mdm2. Taken together, these results suggest that the phosphorylation of the E6 PBM plays an essential role in regulating the transcriptional activity of p53 in the absence of E6AP.

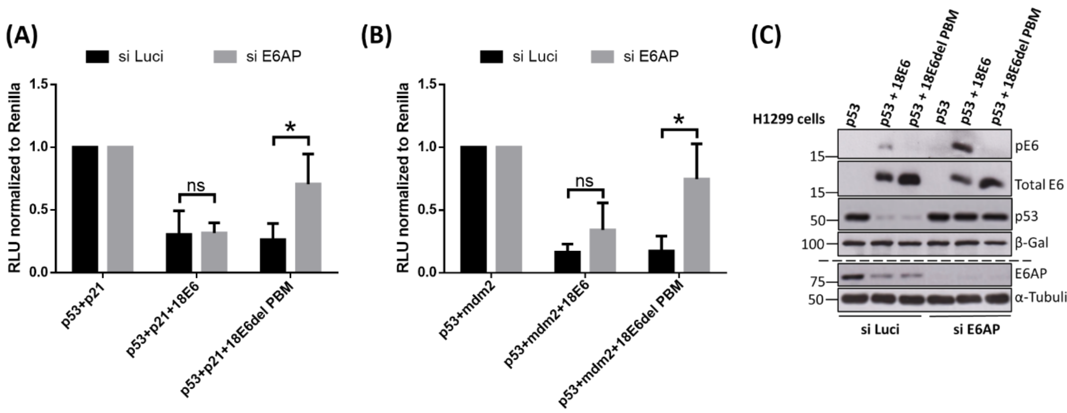


Figure 52. The HPV-18 E6 PBM contributes to inhibition of p53's transcriptional transactivation activity in the absence of E6AP. (A) H1299 cells were transfected with the indicated promoter constructs upstream of a luciferase promoter—p21-Luc (A), Mdm2-Luc (B), together with plasmids expressing p53 and either wild-type HPV-18E6 or mutant HPV-18 E6 delPBM in the presence or absence of E6AP. The histograms show the results from at least three independent experiments quantified using Student's t test; the error bars indicate standard errors of the mean. Also shown are the P values (*, $P < 0.05$; ns, non-significant) for the changes in relative luciferase activity. (C) Corresponding western blot for the luciferase analysis confirms the knockdown of E6AP from the cells and expression of pE6, total E6, p53 and β-gal.

This study demonstrates that the knockdown of E6AP from cervical cancer-derived cells leads to an increase in phosphorylation of the E6 oncoprotein. We show that this phosphorylation of E6 requires p53 transcriptional activity and the enzyme DNA PK. This study therefore defines a feedback loop, whereby activation of p53 can induce phosphorylation of E6 and which, in turn, can inhibit p53 transcriptional activity, independently of E6's ability to target p53 for degradation.

Discussion

Persistent expression of HPV E6 and E7 oncoproteins leads to the development of cancer and is known to be the major hallmark of high-risk HPV-induced carcinogenesis (Smotkin and Wettstein 1986, Androphy, Hubbert et al. 1987, Gaglia and Munger 2018, Thomas and Banks 2018). The high-risk Human Papillomavirus (HPV) E6 oncoprotein is known to contribute to human malignancy by targeting several of its cellular substrates for degradation through the ubiquitin-proteasome pathway (Dukic, Lulic et al. 2020). One of the most important and well-studied functions of E6 is the degradation of the tumour suppressor protein p53 (Scheffner, Werness et al. 1990). This is known to be achieved by its direct interaction with E6AP, a cellular ubiquitin-protein ligase encoded by the UBE3A gene. E6AP acts as a connecting bridge between the E6 and p53 proteins, in turn leading to the poly-ubiquitination of p53 and its subsequent degradation by the 26S proteasome (Scheffner, Werness et al. 1990, Huibregtse, Scheffner et al. 1993, Huibregtse, Scheffner et al. 1993, Scheffner, Huibregtse et al. 1993, Hengstermann, D'Silva M et al. 2005). Apart from p53, E6 is also known to target various other cellular proteins that may also play an essential role in mediating tumorigenesis. These include PDZ domain-containing proteins such as DLG, the MAGI family of proteins and Scribble (Kiyono, Hiraiwa et al. 1997, Gardiol, Kuhne et al. 1999, Nakagawa and Huibregtse 2000, Thomas, Laura et al. 2002, Lee and Laimins 2004, Thomas, Massimi et al. 2005, Handa, Yugawa et al. 2007, Javier 2008). The E6 oncoproteins from high-risk HPV types possess a canonical PBM consensus site, with a significant degree of variation within this site in different HPV E6 types. Through this PBM, E6 has been shown to recognize many different PDZ domain-containing cellular proteins, which are known to be crucial for the development of the cancer and its progression (Watson, Thomas et al. 2003, Lee and Laimins 2004, Pim, Bergant et al. 2012, Vande Pol and Klingelhutz 2013). However, the involvement of E6AP in mediating degradation of these proteins is not yet clear, as there are some scientific reports that show the degradation of these PDZ proteins to be independent of E6AP ubiquitin ligase activity, whilst others show that the degradation of these proteins is E6AP-dependent. Thus, there is still considerable debate as to how much of E6's degradatory activity is independent of E6AP. This is complicated by the fact that E6AP is required for maintaining E6 stability (Tomaic, Pim et al. 2009). When E6AP is removed, E6 turnover is increased and the total E6 levels are greatly reduced. Whilst many targets are subsequently rescued from degradation in the absence of E6AP, it is impossible to determine whether this is due to loss of the E6AP ligase activity, or loss of E6 protein. Thus, to overcome this problem and in order to identify the PDZ proteins that are degraded independently of E6AP, we generated an E6AP knockout cell line using CRISPR/CAS9 (Thatte and Banks 2017), in which we then re-expressed either wildtype E6AP or a catalytically inactive form of E6AP (C833A) (Talis, Huibregtse et al. 1998, Tomaic, Pim et al. 2009) together with the cellular substrates to be screened in the presence of E6. We began our studies by testing the efficiency of this system by performing *in vivo* degradation assays for p53, since its degradation has been shown to be exclusively dependent on the ubiquitin ligase activity of E6AP. Upon analysis, we observed that p53 is not degraded by E6 in the presence of catalytically inactive E6AP, but is degraded in the presence of wildtype E6AP, with E6 protein levels being stabilized in both conditions, as expected and in agreement with the previous studies (Tomaic, Pim et al. 2009), thus validating the competence of our study model.

We then moved forward to screen E6's PDZ domain-containing cellular targets in the presence of wildtype and catalytically inactive E6AP. The results obtained indicate that a subset of E6 targets, including DLG1 and the MAGI family of proteins, appear to be degraded by E6 quite effectively in the presence of catalytically inactive E6AP. In contrast, other E6 targets, such as p53 and Scribble, absolutely require the presence of catalytically active E6AP for E6-mediated degradation to occur (Vats, Thatte et al. 2019). It is interesting that degradation of p53 and Scribble appear to be two characteristics of the high-risk HPV E6 oncoproteins that link particularly closely with cancer-causing ability (Thomas and Banks 2018). It is therefore intriguing that both of these targets also have the absolute requirement for E6AP.

Having shown that DLG1 and the MAGI family of proteins are degraded by E6 independently of the ligase activity of E6AP, we next wanted to determine whether this degradation occurs either through the proteasome degradation machinery or through the lysosomal degradation pathway. Therefore, we performed a MAGI-1 degradation assay in E6AP-knockout HEK293 cells with HPV-18 E6 protein in the presence of proteasome inhibitor, CBZ, or lysosomal protease inhibitor, NH_4Cl. We observed that MAGI-1 protein levels are rescued in the presence of catalytically-inactive E6AP upon treatment with CBZ, compared with the DMSO control. In contrast, treatment with the NH_4Cl did not result in any changes in the protein levels of MAGI-1 in the presence of catalytically inactive E6AP, when compared with the DMSO control, suggesting the involvement of proteasome-mediated ubiquitination pathway, in agreement with the previous studies (Gardiol, Kuhne et al. 1999, Pim, Thomas et al. 2000, Thomas, Laura et al. 2002, Grm and Banks 2004, Massimi, Shai et al. 2008).

These results suggest that E6s have evolved multiple mechanisms of interacting with the proteolytic machinery to bring about the destruction of their target proteins as summarized in figure 53. However, a major question here is how E6 exerts a level of specificity with respect to its substrate proteins: to be degraded in either an E6AP-dependent or -independent manner? Nevertheless, it now remains to be determined which ubiquitin ligase is responsible for the degradation of DLG1 and the MAGI family of proteins in the absence of E6AP.

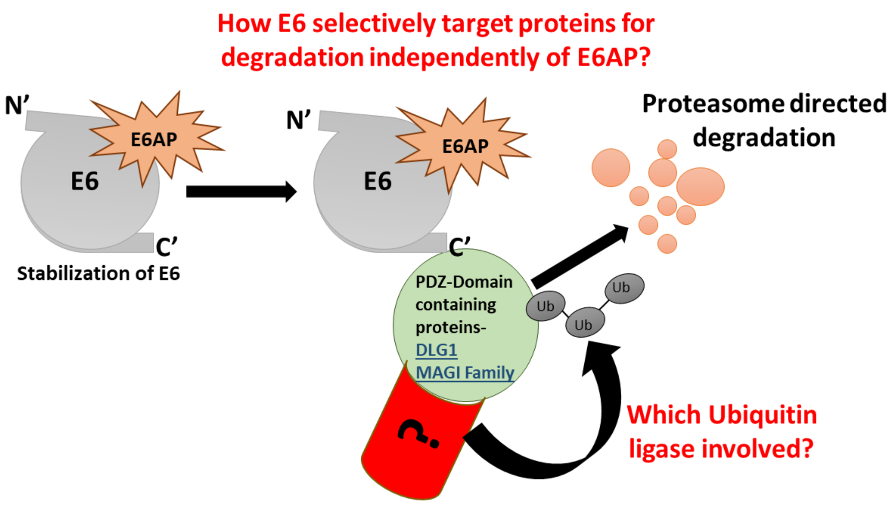


Figure 53. Schematic representation of cellular proteins targeted by E6 independently of E6AP.

Human papillomaviruses (HPVs) have evolved several strategies to utilise the ubiquitin-proteasome system, via the E6 and E7 oncoproteins, to benefit the virus life cycle and induce carcinogenesis (Lou and Wang 2014, Poirson, Biquand et al. 2017, Dukic, Lulic et al. 2020). Both HPV E6 and E7 oncoproteins are unstable and have been reported to be degraded by the ubiquitin-proteasome system (Dukic, Lulic et al. 2020). Intriguingly, HPV E6 oncoprotein is stabilised by its interaction with the E6AP ubiquitin ligase. However, there is no information available about the ubiquitin ligases that regulate the stability and activity of HPV E6 in the absence of E6AP (Tomaic, Pim et al. 2009). We, therefore, became interested in identifying the ubiquitin ligase(s), involved in E6 degradation, when E6AP is absent. In order to identify these, we performed a High-throughput siRNA library screen of approximately 530 human ubiquitin ligases in HEK293 E6AP K/O cells stably expressing GFP-tagged 18E6. We found a total of 11 ubiquitin ligases, whose knockdown increased the GFP-tagged 18E6 fluorescence; and 4 ubiquitin ligases that decreased the GFP-tagged 18E6 fluorescence when they were silenced.

We next validated the 11 ubiquitin ligases whose absence resulted in an increase in the GFP-18E6 levels, in the cervical cancer derived cell line, HeLa. Interestingly, in HeLa cells, of the 11 candidates, silencing of E6AP+FBXO4 gave the maximum increase in the 18E6 protein levels, followed by ablation of E6AP+FBXL19 and E6AP+TRIM69, and then E6AP+BAZ2B, compared with siE6AP. FBXO4 and FBXL19 are the F-box proteins that form functional complexes with Skp1 and CUL to generate the E3 SCF multi-subunit complex (Nguyen and Busino 2020). TRIM69, a member of the tripartite motif (TRIM) family of proteins, is a RING-type E3 ubiquitin ligase, functionally known for its anti-viral defence against various viruses (Rihn, Aziz et al. 2019). BAZ2B belongs to bromodomain gene family that encodes a protein involved in chromatin remodelling (Scott, Guo et al. 2020). Considering these findings, and after finding a marked increase in 18E6 protein levels after FBXO4 knockdown together with E6AP, we decided to concentrate our attention on this ubiquitin ligase for subsequent experiments.

We first showed in an *in vivo* degradation assay, performed in HEK293 E6AP K/O cells, that the ectopic expression of FBXO4 with GFP-tagged 18E6 leads to a decrease in levels of 18E6, suggesting that the presence of FBXO4 increases the degradation of 18E6 in the absence of E6AP. We also validated these results in cervical cancer-derived cell lines, HeLa and C41, and observed similar results, that is silencing of FBXO4 with E6AP leads to increase in the protein levels of 18E6, when compared with siE6AP alone. Having found that FBXO4 targets E6 for degradation in the absence of E6AP, we next wanted to ascertain whether they both interact with each other. To examine this, we performed GST-pulldown and *in vivo* co-immunoprecipitation assays and found that E6 interacts strongly with FBXO4. Since FBXO4 mediates E6 degradation in the absence of E6AP, we next questioned what happens to the E6 and FBXO4 interaction when E6AP is present. To answer this question, we repeated the *in vivo* co-immunoprecipitation assay for E6 and FBXO4 in the presence and absence of E6AP. Interestingly, we observed that the presence of E6AP markedly reduces the interaction between E6 and FBXO4, suggesting that E6AP may compete with FBXO4 to bind with E6, thereby protecting it from undergoing degradation.

To our surprise, we found that the knockdown of E6AP+FBXO4 not only rescued the levels of 18E6, compared with the cells with silenced E6AP alone, but phenotypically also consistently showed a dramatic increase in the number of dead cells. This intrigued us and raised our interest in understanding the possible mechanism behind it. An obvious candidate for this was p53, since not all the p53 in the cell is degraded (Mantovani and Banks 1999) and it is possible that E6/E6AP only targets cellular pools of p53 destined to activate the transcription of downstream pro-apoptotic factors, such as Bax, Fas, PUMAβ, Apaf-1 and PIG (Giampieri, Garcia-Escudero et al. 2004), which are all part of the intrinsic apoptotic pathway. Based on this, we decided to check whether the increased apoptosis upon the removal of E6AP and FBXO4 together from the cervical cancer cells is due to the activation of p53. We found that the knockdown of p53 together with E6AP+FBXO4 led to a marked rescue in the number of live cells when compared with si E6AP+FBXO4; however, the protein levels of E6 remained the same.

Having found that the increase in apoptosis is caused by p53, we next wanted to determine if this phenotype is specific to HPV-positive cancer cell lines. To examine this, we repeated the knockdown assay in HeLa cells (HPV 18 positive), in Normal Immortalized Keratinocytes (NIKS) containing wildtype p53, and in the HPV-negative cervical cancer cell line, C33A, and we determined the number of viable cells through two different assays, including an XTT-cell viability assay and Annexin V exposure apoptosis assay. Results obtained from both assays unequivocally demonstrated that silencing of E6AP+FBXO4 induced apoptosis in HPV-positive HeLa cells in a p53-dependent manner, but had no apparent effect in either NIKS or C33A cells, suggesting that this phenotype is specific to HPV-positive cancer cells. In addition, we observed a substantial decrease in the number of viable cells in all the three cell lines i.e., immortalized NIKS, HeLa and C33A, when FBXO4 expression was depleted. Lee *et al*, 2005 showed that FBXO4 plays a crucial role in telomere maintenance, as its knockdown results in a reduction in the telomere length and reduced cellular proliferation (Lee, Perrem et al. 2006). Since HeLa, C33A and immortalized NIKS all have constitutively active telomerase giving a proliferation advantage to these cells, this may possibly be the reason why we see a reduction in the number of viable cells when FBXO4 expression is ablated from these cells. However, this hypothesis needs to be validated. It is also interesting to note that the amount of apoptosis seen in E6AP-ablated cells is much lower than in cells where both E6AP and FBXO4 are ablated, despite the increased p53 levels in both conditions, with the only differences seen in the E6 protein levels.

This is intriguing, and raises some interesting questions as depicted in Figure 54, such as: do increased E6 protein levels disrupt the equilibrium of the cancer cell, which in turn hyperactivates p53 and pushes the cancer cell towards apoptosis? Is the protection provided by E6AP to E6 a strategy to protect the HPV-transformed cancer cells from dying? Are low-risk HPV type E6 oncoproteins also degraded by FBXO4, since they are also known to be protected by E6AP?

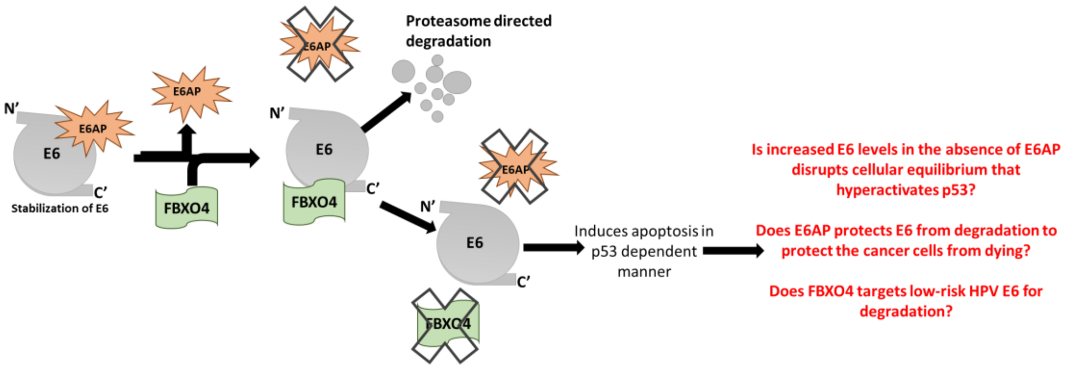


Figure 54. Schematic representation showing FBXO4 mediated degradation of E6 in the absence of E6AP.

Furthermore, this study opens up ways to investigate other possible components of the cellular ubiquitin proteasome pathway that may target E6 for degradation, and also those that E6 might utilize to target its substrates in the absence of E6AP. Current studies are focussed on understanding the mechanistic aspects of p53-induced apoptosis and dissecting the biochemistry between FBXO4 and E6 in the presence and absence of E6AP, mapping their region of interaction, determining the half-life of E6 in the presence of FBXO4, and performing ubiquitination assays. We will also follow up on the other ubiquitin ligases obtained from the screening including both, the ones that increased the expression levels of 18E6 GFP upon their knockdown and also the ones that showed decrease in expression levels of 18E6 GFP when they were silenced.

Like the HPV E6 oncoprotein, E7 is short-lived, having a half-life of approximately 60 minutes (Smotkin and Wettstein 1986, Selvey, Dunn et al. 1994, Dukic, Lulic et al. 2020). Its degradation is controlled by the ubiquitin-proteasome pathway in cervical cancer cells (Reinstein, Scheffner et al. 2000, Wang, Sampath et al. 2001). It has been reported that the first 11 amino acid residues at the N-terminus end of E7 play a crucial role in its ubiquitination, and their deletion leads to the stabilisation of the E7 protein in cells. Moreover, the addition of an N-terminal tag, but not a C-terminal tag, was shown to stabilise the E7 oncoprotein (Selvey, Dunn et al. 1994). In a study of the E7 degradation pathway, Kamaio *et al.*, in 2004, identified an E3 ubiquitin ligase, SOCS1, as an interacting partner of E7, and showed that this interaction results in SOCS-box-dependent degradation of E7. Interestingly, a SOCS1 mutant lacking the SOCS-box can still induce weak E7 ubiquitination, suggesting the role of other ubiquitin ligases in E7 degradation (Kamio, Yoshida et al. 2004). In addition, another group showed that E7 interacts with Cullin1 and Skp2-containing SCF (Skp-Cullin-F-box), an E3 ubiquitin-protein ligase complex, and is ubiquitinated by Cullin 1-containing ubiquitin ligase, both *in vitro* and *in vivo* (Oh, Kalinina et al. 2004). Collectively, these two studies provide insights into the E7 degradation mechanism.

However, knowledge of stabilisation of E7 oncoprotein by ubiquitin ligases is limited. In the case of E7, so far, only USP11 (Ubiquitin specific-protease-11), a deubiquitinase (DUB) enzyme, has been shown to stabilise the E7 oncoprotein by protecting it from ubiquitination (Lin, Chang et al. 2008).

So, during our studies to better understand the role of ubiquitin ligases in HPV-associated cancer, we made the surprising observation that silencing E6AP in cervical cancer-derived SiHa and HeLa cells leads to a dramatic decrease in the levels of E7 oncoprotein. This was intriguing, as E6AP, named E6-associated protein, until now was only known to stabilise HPV E6. Interestingly, it appears that E6AP plays a central role in stabilising both key players, HPV E6 and E7 oncoproteins, which are involved in HPV-associated cancers. We validated these results by exogenously expressing E6AP in HEK293 E6AP knockout cells for high-risk HPV-16 E7 and HPV-18 E7, and low-risk HPV-11 E7. Interestingly, we observed similar patterns and found that the presence of E6AP markedly stabilises not just high-risk E7, but also low-risk HPV 11 E7. Thus, E6AP-mediated stabilisation of E7, like that of E6, is not restricted to cancer-causing HPV types, but is also conserved in non-cancer-causing HPV types. This raises some interesting questions: is the role of E6AP in stabilising HPV E7 evolutionarily conserved; and does E6AP-mediated stabilisation of E7 play any role during the viral life cycle? We also clearly show that the effects on E6AP loss are not at the transcriptional level of E7, but rather at the level of protein stability. Using proteasome inhibitors, we could rescue the levels of E7 expression following ablation of E6AP in cervical tumour-derived cells, and at the same time also demonstrated a marked reduction in the E7 half-life following loss of E6AP. Interestingly, the reduction in the half-life of E7 was also accompanied by increased levels of ubiquitination, suggesting the active involvement of another ubiquitin ligase degrading E7 in the absence of E6AP, in a manner analogous to that observed for E6. Obviously, identifying this ligase is a high priority, and the model developed for E6, involving the expression of a GFP tagged form of the protein in the E6AP KO cells and screening for ligases responsible for the degradation is one clear approach to address this. However, it will be important to ensure that E7 is C-terminally tagged with GFP to avoid interfering with those critical amino terminal residues. As noted above, E7 also associates with a number of known ubiquitin ligases, including cullins 1 and 2, SOCS 1 and p600 and these could also be analysed individually in the absence of E6AP prior to performing the full library screen.

To investigate the possible mechanisms underlying E6AP regulation of E7 we first analysed whether there was any potential for association between E6AP and E7. Using GST pull-down assays and co-immunoprecipitation, we found clear evidence of a potential association between E7 and E6AP, albeit not as strong as that seen between E6 and E6AP. In addition, we show that the C-terminal end of the E7 oncoprotein binds to E6AP more strongly than its N-terminal end, suggesting that the interaction with E6AP is largely mediated by the C-terminal half of E7.

Since pRB and pRB-related family proteins, p107 and p130, are the known primary degradation targets of HPV E7, we next sought to determine whether E6AP-mediated stabilisation of E7 produced a protein that was functionally active with respect to pocket protein degradation. We found again a clear increase in the levels of E7 protein in the presence of E6AP, which also resulted in enhanced levels of degradation of the pocket proteins indicating rescue of a functionally active form of E7.

E6AP has, so far, been primarily considered to be associated with HPV E6, stabilising E6 and also acting with it in carrying out E6's activities. These studies therefore, obviously raise intriguing questions about possible interplay between E6 and E7, with E6AP as a common intermediary. Thus, we analysed what happens to the E6AP-mediated stability of E7 when E6 is present. Surprisingly, we find that in the presence of E6, the E6AP-mediated stabilisation of E7 is significantly reduced. This suggests a possible regulatory loop whereby the levels of E7 expression can be modulated by the levels of E6AP and, by extension, E6. One intriguing observation from this analysis was the apparent ability of E7 to partially overcome the ability of E6 to target E6AP for degradation. Whilst this requires further studies to fully validate, it might also have additional important implications for other aspects of E6 function. This suggests the intriguing possibility that E6 can regulate the stabilisation of E7 by E6AP, and raises some interesting questions that remain unanswered (summarized in Figure 55), such as: do E6 and E7 work together through E6AP? Does E6 play a critical role in maintaining optimal E7 protein levels, perhaps by competing for E6AP binding, E6 can further aid in the survival of the infected cell, since increased E7 levels can induce cell death? Why does E6, after it uses E6AP to degrade its cellular targets, cause E6AP to auto-ubiquitinate? Is it to prevent E6AP stabilising E7 in infected cells? Does E6AP stabilise both HPV E6 and E7 oncoprotein at different times during the viral life cycle? What targets E7 for degradation in the absence of E6AP? In conclusion, this study opens up an unexpected line of further investigation, whereby E6AP would appear to play a key function in regulating both E6 and E7 stability, and raises many important questions about the control and regulation of this possible new auto-regulatory pathway in the HPV life-cycle and in HPV-induced malignancy. Therefore, future studies will be focused on answering some of these questions.

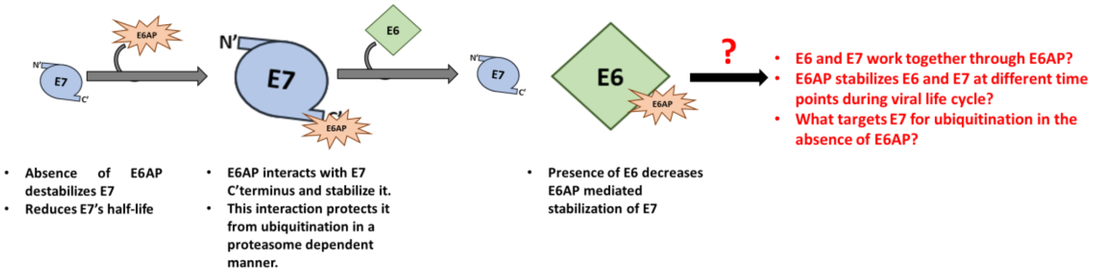


Figure 55. Schematic representation summarizing E6AP mediated stabilization of HPV E7.

The E6 proteins from cancer-causing HPV types, but not from non-oncogenic HPV types, also possess a short carboxy-terminal stretch of amino acids, which confers interaction with cellular proteins that contain a PDZ domain (Scheffner, Huibregtse et al. 1993, Thomas and Banks 2018). Through this PDZ-binding motif (PBM), E6 interacts with various cellular PDZ domain-containing proteins, regulating processes as diverse as cell polarity, cell signalling, cell attachment, and cell proliferation, during both the viral life cycle and cancer progression (Thomas, Narayan et al. 2008, Thomas and Banks 2018). According to previous studies, an intact E6 PBM is required for maintaining the viral episome during the virus life-cycle (Lee and Laimins 2004, Delury, Marsh et al. 2013). In addition, some studies have demonstrated that, in cases where the E6 PBM was mutated, the inactivation of p53 restored the ability of the cell to maintain the HPV genome episomally, thereby suggesting a possible link between the E6 PBM and p53 function (Lorenz, Rivera Cardona et al. 2013, Brimer and Vande Pol 2014). Intriguingly, the E6 PBM is multifunctional, owing to the presence of a phospho-acceptor site embedded within its core. For some time, the phosphorylation of the E6 PBM at the T156 residue was thought to be catalysed mostly by either protein kinase A (PKA) or AKT activity, depending upon the precise amino acid sequence of the E6 PBM. However, more recently it was shown that DNA damage results in a dramatic increase in the levels of phosphorylated E6, which is mediated by the stress-responsive cellular kinases CHK1 and PKA. (Thatte, Massimi et al. 2018). In addition, the retention of an intact PBM and phospho-acceptor site within E6 correlates with the ability of E6 to inhibit p53's transcriptional activation of a subset of p53-responsive promoters, thereby linking regulation of the E6 PBM function with inhibition of p53 activity (Thatte, Massimi et al. 2018).

During the course of our studies to better understand the role of phospho-regulation of E6, we made the surprising observation which is again linked with the loss of E6AP.

We find that ablation of E6AP expression in HeLa cells results in a dramatic increase in the phosphorylation of HPV-18 E6 at this residue. This was somewhat surprising as previous studies had shown that loss of E6AP results in an overall destabilisation of E6, and greatly decreased total levels of E6 protein. Interestingly, the phosphorylated form appears to become the dominant species of E6 under these conditions.

Since one of the major targets of the E6-E6AP complex is p53 (Huibregtse, Scheffner et al. 1991, Huibregtse, Scheffner et al. 1993, Scheffner, Huibregtse et al. 1993, Thomas, Pim et al. 1999), we proceeded to investigate whether the increase in E6 phosphorylation was linked to p53 activity. We found that siRNA ablation of p53 expression, or inhibition of p53 transcriptional activity, both could inhibit the levels of E6 phosphorylation in the absence of E6AP. This indicates that an intact p53 response is required for inducing E6 phosphorylation at T156, which is particularly intriguing as this phosphorylation of E6 has been shown previously to contribute towards E6's inhibition of p53 transcriptional activity, indicating a feedback loop to enhance E6's inhibition of p53.

Having found that, in the absence of E6AP, phosphorylation of HPV-18 E6 takes place in response to p53 activation, we next sought to determine which kinase(s) might be involved in the regulation of E6 phosphorylation. Following from the previous studies (Adhya and Basu 2010, Basukala, Sarabia-Vega et al. 2020), we investigated whether CHK1 or PKA is involved in phosphorylating E6, by repeating the knockdown study in the presence of CHK1 and PKA inhibitors. We show that neither CHK1 nor PKA, in this case, is involved in mediating E6 phosphorylation. To identify the kinase involved, we performed an *in silico* study using the Netphos3.1 software, and identified DNA-dependent protein kinase (DNA PK) as a strong candidate for E6 phosphorylation. Using *in vitro* kinase assays, we found that DNA PK phosphorylates both HPV-18 and HPV-16 E6, and identified T156 as the phospho-acceptor site in HPV-18 E6. Furthermore, we also analysed the phosphorylation of different high-risk HPV types with DNA PK. We observed differences in the phosphorylation patterns of different HPV E6s, with E6s from HPV-16, HPV-18, HPV-31, HPV-39 and HPV-68 being highly phosphorylated, and E6s from HPV-33, HPV-51, HPV-35 and HPV-58 being phosphorylated to a lesser degree. These results highlight the importance of non-canonical residues of the E6 PBM in susceptibility to phosphorylation and how it varies for different kinases. Moreover, they also provide insight into the differential phosphorylation of different high-risk HPV types, depending on the specific amino acids present in the motif.

In order to ascertain whether DNA PK is required for E6 phosphorylation *in vivo*, we included a DNA PK inhibitor in assays of E6 phosphorylation in the absence of E6AP and found a marked inhibition of E6 phosphorylation. Intriguingly, DNA PK phosphorylation of E6 appeared to be specific to conditions where E6AP is lost, and did not occur under other forms of DNA stress response, such as that induced by Teniposide.

We next wanted to ascertain whether phosphorylation of E6 actually requires the transcriptional activity of p53 or whether it simply reflects the p53-dependent activation of the DNA damage stress response pathway. We found that the ablation of E6AP from HeLa cells increases the levels of $^{pS139}\gamma$H2AX, but this drastically decreases upon silencing both E6AP and p53, which was further validated using an inhibitor of p53 transcriptional activity. This suggests that an intact p53 cellular stress response, initiated by upregulation of $^{pS139}\gamma$H2AX, is absolutely essential for phosphorylation of E6 in the absence of E6AP. As noted earlier, this p53-dependent phosphorylation of E6 by DNA PK might indicate the existence of a feedback loop, as shown in Figure 56. Indeed, this would be predicted to result in an increase in pE6 levels, which subsequently inhibit p53's transcriptional activity in a 14-3-3-dependent manner, as demonstrated in previous studies (Boon and Banks 2013, Boon, Tomaic et al. 2015). Using a p21 and mdm2 promoter assay, we find that the ability of E6 to block p53 transcriptional activity in the absence of E6AP is absolutely dependent upon an intact PBM, confirming that phosphorylation of the E6 PBM can inhibit the transcriptional activity of p53, despite the low levels of total E6 protein. This phosphorylation event will then inhibit E6's interaction with PDZ domain-containing substrates, and confer association with 14-3-3 proteins, thereby promoting further inhibition of p53 transcriptional activity; thus, completing a pathway of feedback inhibition.

In summary, this study provides compelling evidence that the high-risk HPV E6 oncoprotein is phospho-regulated by the kinase DNA PK upon the loss of E6AP, in response to p53's transcriptional activation of the DNA damage response pathways and provide mechanistic insights into how the phosphorylation of E6 oncoprotein controls the transcriptional activity of p53 in a negative feedback loop manner.

We now know how E6 utilizes the cell cycle regulatory kinases in a selective manner, presumably to enhance the survival of HPV-transformed cancer cells; but the question arises, when during the viral life cycle is E6 phosphorylated? If the phosphorylation of E6 is dependent on DNA damage, then does the DNA damage caused by E7 oncoprotein in the initial setting of viral propagation, induce E6 phosphorylation? These questions if investigated, would help us to understand when and what role does pE6 plays in the viral life cycle.

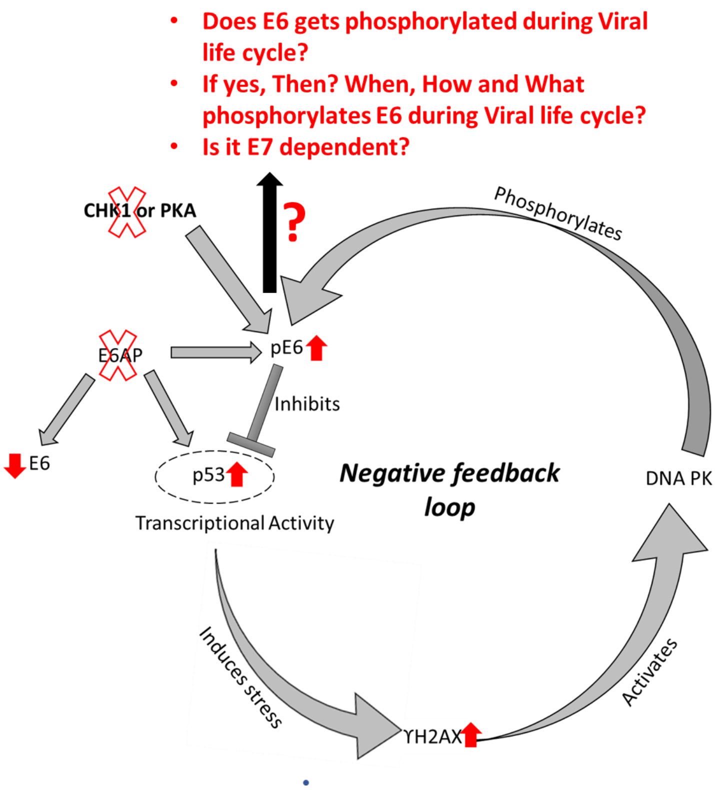


Figure 56. Schematic representation summarizing how E6 gets phosphorylated in the absence of E6AP

Appendix I

Table representing the log fold change (FC) in GFP tagged 18E6 intensity for top 11 ubiquitin ligases (shown in green- increased 18E6 GFP expression when silenced) and 4 ubiquitin ligases (shown in red- decreased 18E6 GFP expression when silenced).

GENE NAME	GENE ID	Round1 Intensity Cell Alexa 488 Mean /MOCK	Round1 LOG2 FC GFP intensity	R1 Z score Fold Intensity	Round2 Intensity Cell Alexa 488 Mean /MOCK	Round2 LOG2 FC GFP intensity	Round2 Z score Fold Intensity
MOCK		1	0	-0.5777785	1	0	-0.518107775
BAZ2B	29994	1.84443222	0.88317677	3.90551394	2.58064169	1.36772985	7.954349526
TRIM54	57159	1.81872352	0.86292624	3.76902029	2.01587102	1.01140333	4.927100875
TRIMl1	339976	1.70205397	0.76727678	3.14959376	1.96892873	0.97741089	4.67548376
RNF5	6048	1.96073691	0.97139597	4.52300333	1.95851701	0.96976166	4.619675485
FBXL19	54620	1.70935131	0.77344894	3.18833712	1.80040103	0.8483183	3.772152063
POS CTRL	CBZ	1.69289014	0.75948836	3.10094084	1.72932842	0.79021188	3.391193074
CUL4B	8450	1.88833163	0.91711215	4.13858638	1.70796201	0.77227588	3.276666157
RNF126	55658	1.59693152	0.67530245	2.59147356	1.70362248	0.76860567	3.25340567
RNF144B	255488	1.66926919	0.73921662	2.97553154	1.6704513	0.74023792	3.075603591
TRIM69	140691	1.51197126	0.59643072	2.14039927	1.64895686	0.72155366	2.960390432
FBX04	26272	1.69260555	0.75924581	3.09942988	1.59475746	0.67333702	2.669874149
UBe2QL1	134111	1.52933959	0.6129088	2.23261188	1.5905143	0.66949334	2.647130253
ZNRF3	84133	0.70643812	-0.5013649	-2.1363687	0.78823518	-0.3433019	-1.653196372
MEX3C	51320	0.74153241	-0.4314184	-1.9500447	0.77424892	-0.3691306	-1.728164666
ASB1	51665	0.66890073	-0.580136	-2.3356637	0.76350016	-0.3892996	-1.785779506
ZNF547	284306	0.73065275	-0.4527422	-2.0078074	0.70764269	-0.498907	-2.085183272

Appendix II

Table representing the log fold change (FC) in GFP tagged 18E6 intensity for all the ubiquitin ligases present in the library screening.

GENE ID	Round 1 Intensity Cell Alexa 488 Mean /MOCK	Round1 LOG2 FC GFP intensity	Z score Fold Intensity	Round 2 Intensity Cell Alexa 488 Mean /MOCK	Round2 LOG2 FC GFP intensity	Z score Fold Intensity
29994	1.844432221	0.883176774	3.90551394	2.580641693	1.367729846	7.954349526
6048	1.96073691	0.971395969	4.52300333	1.958517009	0.969761658	4.619675485
57159	1.818723517	0.862926241	3.76902029	2.015871018	1.011403333	4.927100875
339976	1.702053967	0.767276782	3.14959376	1.968928733	0.977410893	4.67548376
8450	1.888331627	0.917112152	4.13858638	1.707962009	0.772275885	3.276666157
54620	1.709351313	0.773448936	3.18833712	1.800401033	0.848318298	3.772152063
CBZ	1.692890145	0.759488357	3.10094084	1.729328422	0.790211882	3.391193074
255488	1.669269189	0.739216625	2.97553154	1.670451301	0.740237924	3.075603591
55658	1.596931519	0.675302447	2.59147356	1.703622478	0.76860567	3.25340567
26272	1.692605554	0.759245805	3.09942988	1.594757455	0.673337023	2.669874149
140691	1.511971265	0.596430721	2.14039927	1.648956861	0.721553656	2.960390432
134111	1.529339595	0.612908797	2.23261188	1.5905143	0.669493343	2.647130253
653978	1.633191394	0.70769387	2.7839859	1.434886078	0.520936199	1.812941532
652859	1.517852087	0.602031208	2.17162196	1.465413715	0.551308023	1.976573874
55905	1.522951903	0.606870381	2.19869811	1.419186272	0.50506396	1.728788413
84678	1.486213615	0.571641491	2.00364575	1.428863752	0.514868356	1.780661035
643904	1.38319484	0.468004392	1.45669445	1.522741873	0.606671405	2.283860702
94121	1.372054277	0.456337554	1.39754654	1.533473999	0.616803705	2.341386378
9039	1.44682695	0.532892377	1.79453254	1.438939498	0.525005933	1.834668424
92591	1.421362242	0.50727428	1.65933431	1.449711184	0.535765511	1.892406145
23624	1.399084721	0.484483327	1.54105763	1.452289523	0.538329092	1.906226397
5071	1.534687852	0.617945248	2.26100706	1.32037318	0.40094574	1.199136622
11060	1.40269182	0.488198075	1.56020858	1.439755027	0.52582336	1.839039774
287015	1.443818321	0.529889216	1.778559	1.390608639	0.475716458	1.575608362
285533	1.346505383	0.429219998	1.26190136	1.483809616	0.569305995	2.075178439
55274	1.328815144	0.41014042	1.16797965	1.503074369	0.587916393	2.178440173
7862	1.372169168	0.456458355	1.39815653	1.4494879	0.53554329	1.89120931
25827	1.50193982	0.586827008	2.08713993	1.31927998	0.399750768	1.193276917
9666	1.34040995	0.422674301	1.22953925	1.475075884	0.560789175	2.028364435
899	1.498490119	0.583509571	2.06882464	1.317982808	0.398331552	1.186323899
54904	1.406728798	0.492344219	1.58164186	1.394742312	0.479998599	1.597765417
55832	1.521616347	0.605604651	2.19160732	1.279331674	0.355390339	0.979148491
55819	1.477158295	0.562824436	1.95556889	1.305246269	0.384322034	1.118054291

9320	1.423088531	0.509025415	1.66849959	1.354740294	0.438016311	1.383349086
26273	1.457793594	0.543786466	1.85275707	1.316536106	0.396747088	1.178569376
26271	1.445055527	0.53112493	1.78512762	1.327326822	0.408523643	1.236409101
23759	1.415224412	0.50103084	1.62674711	1.347253885	0.430021747	1.343220903
7328	1.379232549	0.463865726	1.4356577	1.377482086	0.462033557	1.505248226
123879	1.543158038	0.625885819	2.3059773	1.216789151	0.283079196	0.643911952
223082	1.272645815	0.347830964	0.86976326	1.456407301	0.542413878	1.928298256
378925	1.405598416	0.491184471	1.57564039	1.305634257	0.384750817	1.120133964
10293	1.32452919	0.405479639	1.1452245	1.384812674	0.469690834	1.54454119
54467	1.320693516	0.401295709	1.12485999	1.370563642	0.454769321	1.468164414
81786	1.413509615	0.499281697	1.61764284	1.273941239	0.349298734	0.950255017
644006	1.279516776	0.355599063	0.90624284	1.401863087	0.487345456	1.635933753
8796	1.370473712	0.454674655	1.38915495	1.291183396	0.368693932	1.042675357
10477	1.419655203	0.505540579	1.65027124	1.220955374	0.288010471	0.666243482
80176	1.369689047	0.453848403	1.38498897	1.262544743	0.336334516	0.889168228
8065	1.452302257	0.538341743	1.82360225	1.190549479	0.25162758	0.503263691
286151	1.287436774	0.364501583	0.948292	1.337650527	0.419701249	1.291745582
5132	1.246512298	0.317897117	0.73101418	1.381481705	0.466216457	1.526686736
115123	1.308330993	0.387727573	1.0592244	1.300898649	0.379508569	1.094750452
25793	1.475481462	0.561185795	1.94666619	1.152091115	0.204254819	0.297121558
118424	1.365352946	0.449273938	1.36196758	1.238892989	0.309051579	0.76239157
149041	1.28614186	0.363049779	0.94141699	1.311933898	0.391695031	1.153900904
115290	1.351738528	0.434816113	1.28968538	1.244981267	0.316124035	0.795025579
26147	1.251433011	0.323581066	0.75713942	1.338376746	0.420484284	1.295638212
51317	1.271589212	0.346632682	0.8641535	1.314317505	0.394313835	1.166677367
7251	1.455584111	0.541598208	1.84102639	1.147495977	0.198489096	0.27249099
8916	1.391749201	0.476899255	1.5021116	1.198717272	0.261491427	0.547044188
7318	1.407548594	0.493184731	1.58599436	1.182955801	0.242396172	0.462560534
55884	1.338613097	0.420739035	1.21999933	1.23806918	0.30809193	0.757975837
120824	1.252886611	0.325255854	0.76485693	1.31735788	0.397647328	1.182974195
652759	1.399149896	0.484550532	1.54140366	1.179240316	0.237857753	0.442645019
9404	1.406952672	0.492573799	1.58283046	1.169843178	0.226315143	0.392275063
54546	1.299522253	0.377981338	1.01245669	1.254153602	0.326714053	0.844190557
51191	1.462750756	0.548683964	1.87907583	1.112993639	0.154445347	0.0875537
114783	1.259509959	0.332862529	0.80002186	1.292488933	0.370151927	1.049673213
644006	1.251089451	0.323184943	0.75531537	1.300876521	0.379484028	1.094631839
378925	1.280559628	0.356774432	0.91177959	1.268471425	0.34309102	0.920936058
54926	1.31306827	0.392941928	1.08437574	1.235362601	0.304934561	0.743468201
7703	1.139060076	0.18784384	0.16052465	1.418589561	0.504457236	1.725589956
6364	1.193065029	0.254672681	0.44724985	1.353922915	0.437145602	1.378967823
29116	1.218456952	0.285055282	0.58206164	1.322187494	0.402926774	1.208861595
23295	1.245164058	0.316335839	0.72385605	1.28858786	0.365790908	1.028762926
8028	1.214519472	0.280385621	0.56115662	1.32047808	0.401060353	1.199698901

51773	1.358011304	0.441495489	1.32298905	1.180574293	0.239488833	0.449795322
84295	1.153434161	0.205935657	0.2368401	1.383496804	0.468319311	1.537487945
7334	1.338544839	0.420665467	1.21963693	1.190685257	0.251792104	0.503991479
153769	1.275298209	0.350834639	0.88384546	1.248592477	0.320302679	0.814382162
79726	1.233455882	0.302706114	0.66169454	1.283569658	0.360161591	1.001864669
27246	1.256388162	0.329282255	0.7834475	1.257578703	0.330648691	0.86254957
390231	1.183934706	0.243589518	0.39877478	1.331058077	0.41257352	1.256409138
399664	1.266567011	0.340923409	0.83748944	1.240269728	0.310653905	0.769771078
150726	1.336779822	0.418761862	1.21026603	1.173685241	0.231045558	0.41286905
26263	1.350815956	0.433831126	1.28478722	1.148072611	0.19921389	0.275581826
57799	1.341775771	0.424143598	1.23679072	1.15478975	0.207630207	0.311586617
282808	1.365744875	0.449688009	1.36404842	1.133786227	0.181148648	0.199004837
54469	1.304888257	0.383926269	1.04094609	1.183428445	0.242972478	0.46509397
8065	1.316426539	0.396627016	1.10220559	1.173052575	0.230267675	0.409477875
26268	1.284589673	0.361307604	0.93317606	1.197839847	0.260435031	0.54234107
64718	1.285800886	0.36266725	0.93960668	1.192052781	0.253448116	0.511321599
170392	1.199107759	0.261961313	0.47933215	1.272827007	0.348036352	0.94428258
55819	1.27190901	0.346995466	0.86585139	1.181311008	0.240388839	0.453744216
7335	1.235225866	0.304774868	0.67109181	1.213569708	0.27925698	0.626655293
26270	1.345137952	0.427754138	1.25464134	1.114250395	0.156073473	0.094290088
399940	1.199618532	0.262575714	0.48204396	1.243085988	0.313926095	0.784866621
59352	1.273382957	0.34866636	0.87367692	1.168758533	0.224976898	0.386461217
55521	1.213353973	0.27900049	0.5549687	1.225109037	0.292910158	0.688507687
26224	1.258111868	0.331260209	0.79259907	1.180767985	0.23972551	0.450833536
5913	1.202012489	0.265451885	0.49475405	1.235091093	0.30461745	0.742012881
373	1.162318797	0.21700582	0.28401076	1.266778591	0.341164391	0.911862239
84961	1.322552414	0.403324898	1.13472932	1.111046148	0.151918741	0.077114879
7681	1.22619267	0.294185685	0.62313241	1.19801302	0.260643588	0.543269303
84085	1.238253417	0.308306602	0.6871658	1.183197655	0.242691098	0.463856901
146330	1.222406095	0.289723641	0.60302858	1.1974625	0.259980477	0.540318439
23133	1.133467387	0.180742881	0.13083172	1.289835913	0.367187545	1.035452663
51257	1.313920908	0.393878435	1.0889026	1.108586767	0.148721691	0.063932259
7763	1.176630823	0.234661734	0.35999672	1.233492391	0.302748817	0.733443621
7332	1.24082834	0.311303542	0.70083668	1.16637765	0.22203498	0.373699357
120824	1.144016728	0.194108148	0.1868407	1.262310934	0.336067321	0.887914982
22888	1.173057613	0.230273871	0.3410257	1.22984814	0.298480184	0.71390993
23291	1.192104095	0.253510218	0.44214802	1.208342709	0.273029688	0.598637855
6045	1.24545076	0.316667985	0.72537822	1.154749725	0.207580203	0.311372077
27154	1.196515226	0.258838756	0.46556777	1.199735472	0.262716343	0.552501879
92979	1.19612413	0.258367116	0.46349135	1.198367409	0.261070294	0.545168874
9810	1.185473708	0.245463666	0.40694571	1.207816591	0.272401395	0.59581779
51366	1.17459471	0.232163045	0.34918651	1.215532703	0.281588709	0.637177216
80028	1.242780659	0.313571694	0.71120201	1.148747106	0.200061228	0.279197214

140456	1.237441147	0.307359912	0.68285327	1.150583896	0.202366183	0.289042659
51230	1.214792629	0.28071006	0.56260687	1.171533453	0.22839815	0.401335171
51255	1.159403921	0.21338327	0.26853499	1.22202924	0.289278805	0.671999548
10193	1.141750574	0.191247515	0.17480915	1.23918577	0.309392482	0.763960912
344558	1.125987417	0.171190706	0.09111878	1.254744245	0.327393329	0.847356483
55072	1.167586094	0.223528934	0.3119761	1.20972638	0.274680771	0.606054526
114783	1.16746861	0.22338376	0.31135235	1.209254492	0.274117897	0.603525141
330	1.216867613	0.283172221	0.57362346	1.159346084	0.213311299	0.336009193
64219	1.178848084	0.237377813	0.3717687	1.196436798	0.258744189	0.534820535
7321	1.108434083	0.148522978	-0.0020761	1.271666537	0.34672041	0.938062303
4193	1.190797058	0.251927562	0.43520865	1.182751979	0.242147575	0.461468017
84282	1.200976723	0.26420819	0.48925493	1.163645453	0.218651556	0.359054407
22823	1.235400022	0.304978261	0.67201645	1.127265441	0.172827272	0.164052527
23014	1.32473017	0.405698531	1.14629155	1.045225481	0.063814201	-0.275692962
326	1.197293769	0.259777177	0.46970124	1.154811991	0.207657993	0.311705832
57484	1.289455945	0.366762483	0.95901226	1.070351791	0.098085043	-0.141012481
5192	1.154443105	0.207197074	0.24219683	1.189250904	0.250053122	0.496303151
50862	1.133309303	0.180541655	0.12999242	1.206403884	0.270712978	0.588245487
254170	1.220828073	0.287860043	0.59465049	1.119074941	0.162306652	0.120150318
84844	1.143759555	0.193783795	0.18547531	1.192908497	0.254483385	0.515908355
9640	1.22930366	0.297841331	0.63964941	1.105101678	0.144179115	0.045251705
55806	1.134600736	0.182184705	0.13684895	1.19369485	0.255434081	0.520123315
10425	1.119772157	0.163205213	0.05812048	1.208109318	0.272751006	0.59738685
26190	1.137237491	0.185533566	0.15084811	1.187180058	0.247538763	0.48520313
9618	1.153328786	0.205803849	0.23628064	1.168363325	0.224488977	0.384342848
59352	1.245167691	0.316340048	0.72387534	1.081022735	0.112396865	-0.083814747
4331	1.112049872	0.15322149	0.01712102	1.206835289	0.271228788	0.590557877
84759	1.255411424	0.328160242	0.77826176	1.068535054	0.095634238	-0.150750439
54850	1.206662304	0.271021981	0.51944104	1.109974394	0.150526396	0.071370134
867	1.164217011	0.219360003	0.29408883	1.148131352	0.199287703	0.275896684
9820	1.24123447	0.311775667	0.70299293	1.074819151	0.104093933	-0.117066812
143279	1.295215313	0.373191947	0.98959012	1.029944717	0.042566901	-0.357599967
57484	1.259907108	0.333317369	0.80213042	1.057113313	0.080130029	-0.211972547
7732	1.111806782	0.152906088	0.0158304	1.196312471	0.258594264	0.534154125
220972	1.096352323	0.132711497	-0.066221	1.212090329	0.277497217	0.618725617
165918	1.144009721	0.194099311	0.1868035	1.159902925	0.214004068	0.338993941
285231	1.213829373	0.279565638	0.55749272	1.091444063	0.126238194	-0.027954994
79444	1.105988194	0.145335985	-0.0150619	1.193074377	0.254683984	0.516797491
64844	1.083599608	0.115831777	-0.1339282	1.209932042	0.274926018	0.607156901
79142	1.205067337	0.269113765	0.51097298	1.086454064	0.119627178	-0.054702075
7128	1.170052025	0.226572679	0.32506831	1.118608275	0.161704907	0.117648922
201456	1.207522108	0.272049603	0.52400594	1.080943469	0.112291075	-0.084239628
157574	1.268534547	0.34316281	0.84793556	1.028551355	0.040613828	-0.36506858

728919	1.102000692	0.14012513	-0.0362325	1.181688678	0.24085	0.455768579
10517	1.261244284	0.334847731	0.80922981	1.031947549	0.045369644	-0.346864511
81545	1.256063065	0.328908901	0.78172148	1.035165949	0.049862067	-0.329613442
84166	1.167221226	0.223078024	0.31003892	1.113545893	0.155161017	0.090513858
9870	1.110112945	0.150706467	0.00683741	1.170630822	0.227286169	0.396496943
3846	1.121499121	0.165428489	0.06728934	1.158092433	0.211750407	0.329289453
652436	1.185529708	0.245531816	0.40724303	1.094617645	0.130427018	-0.010944158
7737	1.104205151	0.143008237	-0.0245285	1.174693946	0.232284927	0.41827585
26994	1.070899157	0.098822633	-0.201358	1.210278371	0.275338913	0.60901327
55743	1.070831189	0.098731065	-0.2017188	1.208240346	0.272907468	0.598089177
9678	1.090504178	0.124995297	-0.0972702	1.185335907	0.245295956	0.475318229
57674	1.109312972	0.149666453	0.00259017	1.162256276	0.216928216	0.351608225
57574	1.004611196	0.006637259	-0.5532966	1.281060182	0.357338253	0.988413534
653111	1.128754419	0.174731635	0.10580945	1.13705477	0.185301748	0.216524678
494188	1.303889402	0.382821503	1.03564293	0.983460465	-0.024061037	-0.606761959
55236	1.073218623	0.101943994	-0.1890434	1.194544464	0.256460555	0.524677361
64326	1.098637375	0.135715277	-0.0540891	1.166311138	0.221952709	0.373342842
114907	1.089580682	0.12377303	-0.1021733	1.175210853	0.232919625	0.421046545
79589	1.100773178	0.138517222	-0.0427496	1.163068049	0.217935509	0.355959441
8452	1.203043184	0.26668843	0.50022626	1.064083186	0.089610939	-0.174613068
57154	1.162374079	0.217074436	0.28430426	1.100728653	0.138458866	0.021811689
152006	1.169339796	0.225694221	0.32128692	1.093083714	0.128403895	-0.019166239
23321	1.219584311	0.286389497	0.58804706	1.047563341	0.06703748	-0.263161711
30827	1.096233661	0.13255534	-0.066851	1.165054146	0.220397006	0.366605196
80853	1.199196797	0.262068435	0.47980487	1.061579997	0.086213092	-0.188030502
135644	1.067854441	0.094715007	-0.2175231	1.190187523	0.251188899	0.501323557
29964	1.085797774	0.118755431	-0.1222576	1.165104412	0.220459249	0.366874627
23220	1.078804978	0.109434083	-0.1593841	1.172643139	0.229764036	0.407283239
166336	1.186529193	0.246747596	0.41254953	1.065782034	0.091912419	-0.165507006
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652591	1.203803638	0.267600082	0.50426369	1.047960396	0.067584196	-0.261033443
10346	1.123250008	0.167679073	0.07658522	1.122110984	0.166215374	0.136423926
22992	1.214394509	0.280237173	0.56049316	1.035315812	0.050070914	-0.328810155
55293	1.213126827	0.278730386	0.55376273	1.033570038	0.047636153	-0.338167746
221687	1.129468432	0.175643949	0.10960032	1.109891624	0.15041881	0.070926472
26269	1.123648371	0.168190636	0.07870022	1.114797736	0.156781977	0.097223908
7317	1.125697718	0.170819475	0.0895807	1.111520971	0.152535167	0.079659996
11059	1.19277197	0.25431826	0.44569393	1.048776533	0.06870731	-0.256658836
9354	1.17983117	0.238580429	0.37698813	1.058188647	0.081596845	-0.206208608
54455	1.158644906	0.212438487	0.26450519	1.073166645	0.10187412	-0.125924475
80128	1.051749049	0.072790514	-0.3030305	1.181332903	0.240415577	0.453861573
8454	1.197889439	0.260494758	0.47286379	1.035641167	0.050524218	-0.32706621
117584	1.030192387	0.042913784	-0.4174799	1.200918684	0.264138467	0.55884406

51136	1.06213492	0.086967039	-0.2478894	1.16382047	0.218868527	0.359992521
79836	1.129392479	0.175546929	0.10919707	1.094140908	0.129798547	-0.013499533
55008	1.171976226	0.228943305	0.33528436	1.047934297	0.067548266	-0.261173336
162333	1.07407347	0.103092681	-0.1845048	1.142587264	0.192304354	0.246179611
64839	1.095295438	0.131320065	-0.0718323	1.119461148	0.162804459	0.122220441
92912	1.139806198	0.188788543	0.16448599	1.074663528	0.103885029	-0.117900977
89870	1.164517159	0.219731897	0.29568239	1.051412879	0.072329311	-0.24252766
7319	1.174800626	0.232415939	0.35027977	1.04114037	0.058164591	-0.29758972
7326	1.192990375	0.254582404	0.4468535	1.023470866	0.033470035	-0.392300699
51444	1.15984734	0.21393493	0.2708892	1.051147842	0.071965596	-0.243948294
375593	1.105409721	0.144581205	-0.0181331	1.100662544	0.138372216	0.021457336
8085	1.086895764	0.120213589	-0.1164281	1.118557539	0.16163947	0.11737697
26260	1.13344256	0.180711281	0.13069991	1.072254468	0.100647328	-0.130813867
7327	1.118565708	0.161650007	0.05171515	1.08597484	0.118990679	-0.057270783
4734	1.230189064	0.298880056	0.64435023	0.986622165	-0.019430397	-0.589814815
84893	1.16095873	0.215316688	0.27678984	1.0452638	0.06386709	-0.275487571
56995	1.134654472	0.182253032	0.13713425	1.067257105	0.093907767	-0.157600421
56852	1.282599173	0.359070382	0.92260802	0.944133212	-0.082937664	-0.817561456
9655	1.152660329	0.204967436	0.23273165	1.043359764	0.061236704	-0.285693462
8284	1.068824653	0.096025189	-0.212372	1.120974836	0.164753893	0.130334019
113878	1.152370165	0.204604215	0.23119109	1.037312865	0.052851093	-0.318105676
30837	1.171420111	0.228258568	0.33233181	1.019759565	0.028229039	-0.412193784
151636	1.12868634	0.174644619	0.10544801	1.058061854	0.081423969	-0.206888238
79097	1.048759663	0.068684104	-0.3189018	1.137408912	0.185751014	0.218422931
84675	1.043846976	0.061910234	-0.3449844	1.140445092	0.189596988	0.234697274
58508	1.100430573	0.138068127	-0.0445686	1.081047349	0.112429713	-0.083682817
257160	1.069431777	0.09684445	-0.2091486	1.112095069	0.153280124	0.082737243
23066	1.100323313	0.137927499	-0.0451381	1.080790974	0.112087531	-0.085057022
126433	1.085357814	0.11817074	-0.1245935	1.095627211	0.131757002	-0.005532747
166655	1.006751161	0.009707136	-0.541935	1.179281617	0.237908281	0.442866401
11065	1.17072774	0.227405607	0.32865585	1.013321125	0.019091441	-0.446704711
9830	1.162497503	0.217227617	0.28495955	1.019079584	0.027266722	-0.415838574
51132	1.10719808	0.146913346	-0.0086383	1.069764174	0.097292795	-0.144162186
27072	1.15733552	0.210807173	0.25755335	1.021597376	0.030826725	-0.402342863
9690	1.134951512	0.182630663	0.1387113	1.037648469	0.053317775	-0.316306792
5896	1.082665231	0.114587218	-0.1388891	1.084773376	0.117393675	-0.063710799
4850	1.08040773	0.111575867	-0.1508747	1.085603663	0.118497494	-0.059260342
57531	1.08887807	0.122842414	-0.1059036	1.073886084	0.102840963	-0.12206818
54476	1.08200018	0.113700739	-0.14242	1.08026869	0.111390192	-0.087856534
222484	1.100875575	0.138651419	-0.042206	1.061594884	0.086233323	-0.18795071
254225	0.991741822	-0.011963499	-0.6216232	1.177688476	0.235957966	0.434326945
283450	1.136842983	0.185033008	0.14875357	1.025729317	0.036650064	-0.380195089
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164832	1.005898591	0.008484868	-0.5464615	1.158070515	0.211723102	0.329171971
7128	1.158280085	0.211984155	0.26256827	1.005209062	0.007495582	-0.490186485
267	1.137108123	0.185369441	0.15016127	1.019789529	0.02827143	-0.412033172
89970	1.033107218	0.046989988	-0.4020044	1.122255333	0.166400952	0.137197659
83737	1.113059921	0.154531261	0.02248361	1.040626303	0.057452079	-0.30034519
79791	1.093313153	0.128706686	-0.0823567	1.058123891	0.081508557	-0.206555707
8651	1.089877309	0.124165735	-0.1005984	1.059919601	0.083954835	-0.196930455
7322	1.142414607	0.19208633	0.17833465	1.009850622	0.014141905	-0.465307081
64324	1.034299831	0.048654465	-0.3956725	1.11470308	0.156659475	0.096716541
26235	1.122727254	0.167007495	0.0738098	1.025787455	0.036731832	-0.379883465
122809	1.02073937	0.029614544	-0.4676683	1.127134724	0.172659968	0.163351866
148581	1.166215117	0.221833929	0.30469725	0.985093745	-0.021667072	-0.598007356
57649	1.043419569	0.061319397	-0.3472537	1.09985256	0.137310137	0.017115706
648	1.063962643	0.089447498	-0.2381856	1.077395601	0.107548079	-0.10325669
79960	1.063862365	0.089311517	-0.238718	1.076990552	0.107005594	-0.105427806
29128	1.034078738	0.048346041	-0.3968464	1.103899416	0.142608724	0.038807413
162517	1.035307459	0.050059273	-0.3903228	1.102269307	0.140476747	0.030069806
140458	1.238739728	0.308873095	0.68974775	0.919511812	-0.121059988	-0.949535555
142685	0.959689078	-0.05936102	-0.7917988	1.184876413	0.244736588	0.472855275
79596	1.027330226	0.038899997	-0.4326758	1.102633472	0.140953302	0.03202178
142686	1.108587785	0.148723016	-0.00126	1.018767681	0.026825097	-0.417510421
399937	1.033174114	0.047083402	-0.4016492	1.091717708	0.126599858	-0.026488221
142684	1.087796005	0.121408033	-0.1116486	1.035812467	0.050762827	-0.326148018
653192	1.021563547	0.03077895	-0.4632925	1.102854339	0.141242257	0.033205654
54461	0.967088447	-0.048280254	-0.7525138	1.163321019	0.218249264	0.357315392
5253	0.978342572	-0.031588374	-0.692763	1.148798504	0.200125775	0.27947271
56254	0.991148629	-0.01282668	-0.6247726	1.131526723	0.178270657	0.186893585
9025	1.029272322	0.041624738	-0.4223648	1.088612178	0.12249008	-0.043134289
26261	1.138753847	0.187455927	0.15889881	0.983484519	-0.024025751	-0.606633027
26001	1.112486062	0.153787261	0.01943686	1.005420411	0.007798883	-0.489053623
55223	1.091157818	0.125859779	-0.0937999	1.021459883	0.030632544	-0.403079847
494470	1.01206386	0.017300325	-0.5137286	1.100008664	0.137514886	0.017952444
89910	1.115812018	0.158093995	0.03709515	0.997655513	-0.00338635	-0.530674546
196346	1.064131409	0.08967632	-0.2372895	1.045819051	0.064633256	-0.272511349
283116	1.115498869	0.157689051	0.03543257	0.996669137	-0.004813441	-0.535961661
55208	1.06187931	0.086619803	-0.2492465	1.046952326	0.06619575	-0.266436834
642678	1.158275667	0.211978653	0.26254482	0.957970182	-0.061947344	-0.743393392
554251	1.088321117	0.122104297	-0.1088606	1.019253763	0.027513283	-0.414904953
57630	1.067353402	0.094037933	-0.2201832	1.038441817	0.054420386	-0.312054337
7325	1.122372652	0.166551761	0.07192713	0.987307347	-0.018428833	-0.586142146
642446	0.995452178	-0.006576086	-0.601924	1.11273031	0.154103972	0.08614222
391712	1.038042704	0.053865796	-0.3758007	1.063964816	0.089450443	-0.175247547
200312	1.075814133	0.105428847	-0.1752632	1.026486452	0.037714586	-0.376136741

56957	1.007025002	0.010099503	-0.5404811	1.095839444	0.132036438	-0.004395148
23194	1.102327851	0.14055337	-0.0344955	1.000165069	0.000238125	-0.517222982
7336	1.026709689	0.038028305	-0.4359704	1.073339168	0.10210603	-0.12499973
23142	1.101146682	0.139006661	-0.0407666	1.000631701	0.000911064	-0.514721769
10155	0.965964291	-0.049958238	-0.7584822	1.137884787	0.186354489	0.220973684
140739	1.044526857	0.062849587	-0.3413748	1.052030855	0.073177018	-0.239215221
80230	1.018472304	0.026406748	-0.4797047	1.077861321	0.108171571	-0.100760364
7267	1.061765666	0.086465396	-0.2498498	1.033819007	0.047983632	-0.336833236
8315	1.045878614	0.06471542	-0.334198	1.04878423	0.068717898	-0.25661758
51105	1.10765633	0.147510329	-0.0062053	0.989354963	-0.015439867	-0.575166639
57448	1.15694995	0.210326455	0.25550627	0.945638582	-0.080639196	-0.809492464
200933	1.049759277	0.070058537	-0.3135946	1.042166838	0.059586253	-0.292087714
85451	1.000802991	0.001158007	-0.5735153	1.092546876	0.12769518	-0.022043768
55030	1.002641058	0.003805219	-0.5637565	1.088363492	0.122160469	-0.044467281
9781	0.984784588	-0.022119911	-0.6585608	1.106148934	0.145545645	0.050865137
115992	1.000691554	0.000997357	-0.5741069	1.084847599	0.117492384	-0.063312953
8193	1.032748452	0.046488897	-0.4039092	1.048792872	0.068729786	-0.256571256
201292	1.013788025	0.019756028	-0.5045746	1.066391781	0.092737566	-0.162238679
7323	1.049883786	0.070229641	-0.3129336	1.027727161	0.039457312	-0.369486368
10668	1.015173262	0.021725977	-0.4972201	1.061903211	0.086652276	-0.186298032
23608	1.02418036	0.034469798	-0.4493992	1.049369749	0.069523106	-0.253479115
142678	0.977429291	-0.032935757	-0.6976118	1.098894472	0.136052849	0.011980222
9978	1.170977909	0.227713859	0.32998405	0.917010661	-0.124989589	-0.96294207
196403	1.020785263	0.029679406	-0.4674246	1.050700784	0.071351881	-0.246344583
331	0.979982371	-0.029172299	-0.6840569	1.09432179	0.130037031	-0.012529984
84108	1.023337943	0.033282654	-0.4538718	1.047789378	0.067348742	-0.261950122
10336	1.061400478	0.085969103	-0.2517887	1.009597468	0.013780197	-0.466664026
11043	0.989158028	-0.01572707	-0.6353411	1.082245811	0.114028217	-0.077258891
4302	1.037530469	0.053153704	-0.3785203	1.029255651	0.04160137	-0.361293453
91445	0.991399722	-0.012461241	-0.6234395	1.076046725	0.105740725	-0.110486849
10210	1.042146693	0.059558367	-0.3540117	1.021953874	0.031330081	-0.400431986
4591	0.963403423	-0.053788044	-0.7720785	1.104316912	0.143154249	0.041045247
27339	0.999909528	-0.000130529	-0.5782589	1.063788365	0.089211163	-0.176193346
7844	1.001218263	0.001756513	-0.5713105	1.061204784	0.085703084	-0.190041699
55159	0.97871702	-0.031036307	-0.690775	1.08460222	0.117166028	-0.064628217
84259	1.091744924	0.126635823	-0.0906828	0.967134442	-0.048211641	-0.694271694
10739	1.065221138	0.091152962	-0.2315039	0.990139631	-0.014296105	-0.570960713
168433	1.096513176	0.132923148	-0.065367	0.957722482	-0.062320426	-0.744721098
23338	0.953283324	-0.069023035	-0.8258085	1.100927636	0.138719643	0.022878261
144165	0.971556894	-0.041629613	-0.7287898	1.079419635	0.110255835	-0.092407589
25820	1.076536383	0.106397079	-0.1714286	0.973306032	-0.039034598	-0.66119112
148066	1.071737374	0.099951421	-0.1969077	0.976864621	-0.033769454	-0.642116592
220441	1.028904116	0.041108543	-0.4243197	1.01716953	0.024560152	-0.426076725

29089	1.087887541	0.121529427	-0.1111626	0.960116664	-0.058718376	-0.731887953
22838	1.076149809	0.105878927	-0.173481	0.970090103	-0.043809343	-0.678428947
23403	1.012685223	0.018185806	-0.5104296	1.030657381	0.04356482	-0.35377999
148479	1.001950803	0.002811672	-0.5674212	1.03776147	0.053474877	-0.315701093
652433	1.009434685	0.013547565	-0.5276875	1.02973704	0.04227597	-0.35871314
580	0.995674731	-0.006253578	-0.6007424	1.041778267	0.059048246	-0.294170507
55167	0.974617926	-0.037091337	-0.712538	1.06382452	0.089260195	-0.175999552
26038	0.963504667	-0.053636441	-0.771541	1.075316388	0.104761203	-0.114401556
158506	1.002597935	0.003743168	-0.5639855	1.031267694	0.044418872	-0.350508628
57534	1.02049324	0.029266626	-0.468975	1.008955378	0.012862371	-0.470105716
9757	0.984522869	-0.022503377	-0.6599503	1.044586914	0.062932536	-0.279115771
23650	0.988397441	-0.01683682	-0.6393793	1.040269707	0.05695762	-0.302256594
9246	1.071588484	0.099750982	-0.1976982	0.959231569	-0.060048955	-0.736632182
55823	1.010276694	0.014750471	-0.5232171	1.017255	0.024681372	-0.425618595
27309	0.967124272	-0.048226812	-0.7523236	1.060549952	0.084812574	-0.193551686
117854	0.99220201	-0.011294215	-0.6191799	1.032930831	0.046743649	-0.341593983
7468	1.030499925	0.0433444	-0.4158471	0.99273034	-0.01052621	-0.557074153
79754	1.1072596	0.146993505	-0.0083117	0.923860969	-0.114252337	-0.926223473
9616	0.981112918	-0.027508907	-0.6780546	1.03944623	0.055815131	-0.306670545
25831	1.04530453	0.063923306	-0.3372459	0.975018981	-0.03649779	-0.652009478
284996	1.112398058	0.153673132	0.01896963	0.915691466	-0.127066519	-0.970013138
115426	1.15795657	0.211581146	0.26085065	0.87946921	-0.185295025	-1.16416942
55284	1.033142218	0.047038863	-0.4018186	0.984295246	-0.022836969	-0.602287422
51592	1.061674355	0.08634132	-0.2503346	0.957358044	-0.062869513	-0.746674534
54165	1.095726835	0.131888179	-0.0695419	0.924633346	-0.113046701	-0.922083424
3670	0.975947304	-0.035124843	-0.70548	1.036466656	0.051673705	-0.322641471
142689	1.100222956	0.13779591	-0.0456709	0.917556192	-0.124131582	-0.960017946
22954	1.041554909	0.058738898	-0.3571536	0.968901013	-0.045578813	-0.68480263
8945	1.050642832	0.071272305	-0.3089036	0.960413926	-0.058271772	-0.730294588
138065	1.00009633	0.000138968	-0.5772671	1.008449469	0.012138796	-0.472817455
729974	0.997570977	-0.003508602	-0.5906748	1.009955153	0.014291232	-0.464746781
140460	0.982755993	-0.025094838	-0.6693311	1.024810734	0.035357492	-0.38511882
26231	1.05698983	0.079961495	-0.2752059	0.952320106	-0.070481503	-0.773678577
54708	1.013694913	0.019623517	-0.505069	0.990683363	-0.013504071	-0.568046234
26234	1.103658273	0.142293538	-0.027432	0.908490936	-0.138455974	-1.008608968
7188	0.947378595	-0.077987019	-0.8571581	1.058349379	0.081815964	-0.205347061
130507	1.037781157	0.053502247	-0.3771893	0.965972139	-0.049946517	-0.700501801
9031	0.971209572	-0.042145455	-0.7306338	1.031975444	0.045408642	-0.346714988
MOCK	1	0	-0.5777785	1	0	-0.518107775
7739	0.981359686	-0.027146088	-0.6767444	1.016656196	0.023831884	-0.428828268
26232	0.967665215	-0.047420092	-0.7494516	1.030341056	0.043121967	-0.355475531
80352	0.975497012	-0.035790642	-0.7078707	1.016936727	0.024229918	-0.427324587
26223	1.053899274	0.075736989	-0.2916144	0.938169672	-0.092079231	-0.849526854

63891	1.017347662	0.024812781	-0.4856756	0.970068459	-0.043841531	-0.67854496
4194	1.016015751	0.022922768	-0.4927471	0.970530923	-0.043153915	-0.67606609
79102	0.955896147	-0.065074209	-0.8119364	1.029485828	0.041923969	-0.360059675
151525	0.932220117	-0.101257449	-0.9376381	1.053702931	0.075468187	-0.230252663
440456	0.928153829	-0.107564162	-0.959227	1.058110647	0.081490498	-0.206626699
26001	0.981406952	-0.027076603	-0.6764935	1.000202042	0.000291456	-0.517024801
51725	1.027566938	0.039232378	-0.4314191	0.954908183	-0.066566074	-0.759806128
81844	0.994569758	-0.007855531	-0.606609	0.986325924	-0.019863641	-0.591402704
4281	0.976496523	-0.034313188	-0.7025641	1.00378662	0.005452621	-0.497810968
7187	0.950982482	-0.072509329	-0.8380242	1.030653951	0.04356002	-0.353798373
22893	1.005548588	0.007982794	-0.5483197	0.972215678	-0.040651696	-0.66703557
646754	0.931585603	-0.102239751	-0.9410069	1.049045247	0.069076904	-0.255218494
51127	0.94608816	-0.079953469	-0.8640094	1.032402216	0.046005143	-0.344427434
283807	0.960870104	-0.057586683	-0.7855285	1.014710035	0.021067519	-0.439259963
23609	0.948392846	-0.076443315	-0.8517732	1.027310265	0.038871966	-0.371720989
997	1.03114175	0.044242673	-0.4124395	0.94296172	-0.08472889	-0.823840816
56163	0.972295132	-0.040533797	-0.7248703	0.998180115	-0.00262793	-0.527862607
55258	0.895019216	-0.160009438	-1.1351466	1.080535609	0.111746617	-0.086425812
23072	1.057052573	0.080047132	-0.2748728	0.913596498	-0.130370975	-0.981242454
84447	0.930019192	-0.104667607	-0.9493234	1.036801766	0.05214008	-0.32084524
23024	0.988372697	-0.016872937	-0.6395107	0.975528105	-0.035744657	-0.649280502
84676	1.125146244	0.170112531	0.08665279	0.852416174	-0.230370128	-1.309177421
51529	0.835064786	-0.260039965	-1.453459	1.146678464	0.197460908	0.268109008
57617	0.938161501	-0.092091796	-0.9060939	1.020245002	0.028915643	-0.409591775
10738	0.973804035	-0.038296616	-0.7168592	0.981596194	-0.02679844	-0.61675471
57117	1.02559362	0.036459192	-0.4418959	0.93124315	-0.102770186	-0.886653964
143384	1.024259769	0.034581652	-0.4489776	0.930855029	-0.103371594	-0.888734346
57661	0.966816224	-0.048686411	-0.7539591	0.984260459	-0.022887958	-0.602473886
26046	0.989912787	-0.014626668	-0.631334	0.960539595	-0.058083009	-0.729620982
8453	0.902027849	-0.14875612	-1.0979361	1.052608086	0.073968383	-0.236121182
26091	1.025948851	0.036958807	-0.4400099	0.925063161	-0.112376222	-0.919779558
5977	0.997284578	-0.003922854	-0.5921954	0.95118903	-0.072196018	-0.779741301
168433	1.058605779	0.082165434	-0.2666264	0.894433688	-0.160953568	-1.083957754
1154	1.068557945	0.095665144	-0.213788	0.88567888	-0.175144379	-1.130884734
222235	1.038014804	0.053827019	-0.3759488	0.907046034	-0.140752323	-1.01635384
6047	0.963371446	-0.053835931	-0.7722483	0.975967266	-0.035095334	-0.646926538
25898	0.937970914	-0.092384909	-0.9071058	1.00167234	0.002410662	-0.5091438
54542	0.95674728	-0.063790201	-0.8074176	0.98177387	-0.026537325	-0.615802345
29951	0.95308831	-0.069318199	-0.8268439	0.979708232	-0.029575932	-0.626874447
127544	0.899763759	-0.152381835	-1.1099567	1.035660791	0.050551556	-0.326961021
91694	0.974891978	-0.036685723	-0.711083	0.955847603	-0.065147477	-0.754770707
130888	0.981668404	-0.026692314	-0.6751054	0.948494241	-0.076289081	-0.79418574
10616	0.905011161	-0.143992511	-1.082097	1.028148062	0.040048039	-0.367230285

51666	0.993731549	-0.009071926	-0.6110592	0.936307721	-0.09494534	-0.859507166
26233	1.024791329	0.035330175	-0.4461554	0.907440335	-0.140125307	-1.014240333
9921	0.926478251	-0.110170985	-0.9681231	1.001350928	0.001947662	-0.510866615
140545	0.998229107	-0.002557124	-0.5871806	0.923386653	-0.114993216	-0.928765872
10206	0.953954885	-0.068007056	-0.822243	0.963083594	-0.054267069	-0.715984801
11342	1.001146955	0.001653759	-0.5716891	0.915785695	-0.126918065	-0.969508056
144699	0.963055054	-0.054309822	-0.7739281	0.950735254	-0.072884437	-0.782173602
9604	0.856867722	-0.222855587	-1.337702	1.066932791	0.093469299	-0.159338792
92312	0.98578713	-0.020651949	-0.6532381	0.926547884	-0.110062559	-0.911821241
26145	0.935864561	-0.095628337	-0.9182889	0.971274624	-0.042048825	-0.672079748
5252	0.915372454	-0.127569216	-1.0270864	0.988824721	-0.016213283	-0.578008809
9148	0.93444479	-0.097818667	-0.9258268	0.967777099	-0.047253294	-0.690826963
197131	0.950318331	-0.073517237	-0.8415504	0.950658943	-0.073000241	-0.782582641
154214	0.945819346	-0.080363444	-0.8654366	0.953763881	-0.068295947	-0.765939746
8925	0.981397235	-0.027090888	-0.676545	0.918217718	-0.123091824	-0.956472077
23598	0.943696757	-0.08360475	-0.8767059	0.952347354	-0.070440224	-0.773532522
6049	0.93433435	-0.097989187	-0.9264132	0.961558353	-0.056553683	-0.7241603
7189	0.977690972	-0.032549563	-0.6962225	0.918669685	-0.122381872	-0.95404947
26267	0.991444971	-0.012395395	-0.6231992	0.905120577	-0.1438181	-1.026674559
149603	0.895587961	-0.15909296	-1.132127	1.001861339	0.002682849	-0.50813074
55336	1.007909307	0.011365829	-0.5357861	0.886373661	-0.174013083	-1.12716061
55585	1.011384641	0.016331775	-0.5173347	0.882802366	-0.179837598	-1.146303242
7337	0.951026336	-0.072442802	-0.8377914	0.937827304	-0.092605812	-0.851361991
25820	0.918168146	-0.123169714	-1.0122434	0.970731953	-0.042855114	-0.674988542
112401	0.914240259	-0.129354745	-1.0330975	0.972614311	-0.040060276	-0.664898842
140432	0.896957268	-0.156888839	-1.124857	0.989460928	-0.015285356	-0.574598654
6737	0.912889427	-0.131487969	-1.0402694	0.972154437	-0.040742575	-0.667363828
378884	0.909434656	-0.136958112	-1.0586116	0.974189681	-0.037725393	-0.65645464
493829	0.85857615	-0.219981997	-1.3286315	1.029620093	0.042112113	-0.359339994
23113	1.03419935	0.048514303	-0.396206	0.852436271	-0.230336114	-1.309069699
257218	0.937498019	-0.093112454	-0.9096165	0.938423029	-0.091689676	-0.848168823
10299	0.946582041	-0.079200543	-0.8613872	0.928782446	-0.10658739	-0.89984368
5828	0.919208215	-0.121536403	-1.0067214	0.956185916	-0.064636939	-0.752957302
64400	1.023043578	0.0328676	-0.4554347	0.857927702	-0.221072019	-1.279634872
94120	0.85478405	-0.226368107	-1.3487647	1.026111943	0.037188129	-0.378144163
221656	0.893972228	-0.161698081	-1.1407053	0.979374505	-0.030067454	-0.628663268
129868	0.940969884	-0.087779545	-0.8911835	0.929237665	-0.105880462	-0.89740364
51283	0.939456851	-0.090101195	-0.8992166	0.930633108	-0.103715581	-0.889923873
148066	0.960243845	-0.058527284	-0.7888535	0.910335078	-0.135530422	-0.998724115
54778	0.917016955	-0.124979686	-1.0183553	0.952941752	-0.069540062	-0.77034647
65264	0.931734665	-0.102008926	-0.9402155	0.935135981	-0.096751929	-0.865787857
83856	0.892237671	-0.164500034	-1.1499145	0.976198994	-0.03475283	-0.645684449
84206	0.91159015	-0.133542759	-1.0471675	0.954789972	-0.066744681	-0.760439757

23355	0.878879079	-0.18626341	-1.2208384	0.989562574	-0.015137158	-0.574053817
10765	0.923171942	-0.115328718	-0.985677	0.938123215	-0.092150674	-0.849775872
6468	0.970439115	-0.043290393	-0.7347243	0.8909121	-0.166644997	-1.102833952
51465	0.924969584	-0.112522169	-0.9761329	0.930224505	-0.10434915	-0.892114046
84851	0.921016523	-0.118701056	-0.9971207	0.930123032	-0.104506533	-0.89265795
642219	0.880369854	-0.18381835	-1.2129236	0.971300014	-0.042011112	-0.671943653
672	0.883801848	-0.178205147	-1.1947023	0.964884414	-0.051571967	-0.706332157
401036	0.918036311	-0.123376877	-1.0129433	0.926708769	-0.109812072	-0.910958875
54941	0.8973923	-0.156189289	-1.1225473	0.946412128	-0.079459534	-0.805346155
9306	0.941024414	-0.087695943	-0.890894	0.899554557	-0.152717313	-1.056509193
11074	0.866060172	-0.20746083	-1.2888971	0.976822373	-0.033831851	-0.642343052
140825	0.944300021	-0.082682793	-0.873503	0.892503014	-0.164071054	-1.094306435
79845	0.913567456	-0.130416836	-1.0366696	0.921197609	-0.118417429	-0.940499453
9767	0.908284124	-0.138784432	-1.06472	0.925332273	-0.111956586	-0.918337081
5988	0.879822555	-0.184715509	-1.2158293	0.951513501	-0.071703968	-0.778002092
6596	0.98712942	-0.01868885	-0.6461115	0.847806186	-0.238193603	-1.333887594
64320	0.961085471	-0.057263357	-0.7843851	0.866410674	-0.206877078	-1.234164971
84937	0.868301486	-0.203732041	-1.2769974	0.952833188	-0.069704431	-0.770928389
55632	0.838017952	-0.254946945	-1.4377799	0.986836904	-0.019116427	-0.588663785
91107	0.869102192	-0.202402271	-1.2727463	0.95092481	-0.072596824	-0.781157557
25897	0.889957074	-0.168192344	-1.1620227	0.926161412	-0.110664446	-0.913892784
93611	1.004489462	0.006462429	-0.5539429	0.82033181	-0.285720522	-1.481154032
140459	0.930292658	-0.104243454	-0.9478715	0.883896246	-0.178051063	-1.140439896
10966	0.835805598	-0.258760674	-1.4495258	0.97421199	-0.037692356	-0.656335064
55298	0.843172164	-0.246100856	-1.410415	0.962046831	-0.05582097	-0.721541989
445372	0.904524054	-0.144769226	-1.0846831	0.892093418	-0.164733302	-1.096501927
5071	0.906082615	-0.142285496	-1.0764084	0.875950003	-0.191079567	-1.183032852
23327	0.934900775	-0.097114841	-0.9234059	0.846048512	-0.241187706	-1.343308968
84900	0.819392889	-0.287372722	-1.5366648	0.963334962	-0.053890569	-0.714637433
51619	0.921860526	-0.117379602	-0.9926397	0.849702314	-0.234970601	-1.323724082
55294	0.835390552	-0.259477268	-1.4517294	0.933986136	-0.098526961	-0.871951186
84333	0.90164602	-0.149366942	-1.0999633	0.86439387	-0.210239254	-1.24497532
10771	0.875894668	-0.191170708	-1.2366834	0.887303145	-0.172501013	-1.12217845
146310	0.814524707	-0.295969635	-1.5625112	0.949768748	-0.07435181	-0.787354209
284996	0.895998562	-0.158431678	-1.129947	0.854467317	-0.226902784	-1.298183011
55128	0.80830414	-0.307029858	-1.5955377	0.945904897	-0.080232955	-0.808064979
79654	0.931587589	-0.102236676	-0.9409964	0.815810902	-0.293693308	-1.505386722
56658	0.890713295	-0.166966966	-1.1580078	0.847998821	-0.237865836	-1.33285504
131405	0.864360356	-0.21029519	-1.2979218	0.869318567	-0.202043136	-1.218578262
93082	0.834227433	-0.26148734	-1.4579047	0.894528495	-0.160800655	-1.083449575
90933	0.828603105	-0.271246869	-1.4877656	0.894268757	-0.161219622	-1.08484181
868	0.839703542	-0.252048021	-1.4288307	0.874699224	-0.193141081	-1.189737202
4008	0.801905246	-0.318496319	-1.6295109	0.909474982	-0.136894143	-1.003334349

440730	0.824826409	-0.27783757	-1.507817	0.87126568	-0.19881538	-1.208141471
92369	0.847331323	-0.239001894	-1.388333	0.838811123	-0.253582102	-1.382102366
25893	0.766734514	-0.383200972	-1.8162407	0.907204591	-0.140500154	-1.015503955
11237	0.862274366	-0.213781103	-1.3089968	0.798618776	-0.324421104	-1.597538888
10273	0.860755481	-0.216324633	-1.317061	0.794001285	-0.332786753	-1.622289276
84219	0.801474173	-0.319272063	-1.6317996	0.836368609	-0.257789179	-1.395194579
1840	0.779456955	-0.359458741	-1.7486943	0.8569878	-0.222653428	-1.284672875
84261	0.864097422	-0.210734118	-1.2993178	0.751525849	-0.412105367	-1.849963464
23087	0.746669212	-0.42145885	-1.9227722	0.825523887	-0.276618134	-1.453323785
153830	0.716406446	-0.481149776	-2.0834445	0.847111465	-0.239376279	-1.33761139
51320	0.741532407	-0.431418351	-1.9500447	0.774248921	-0.369130627	-1.728164666
84133	0.706438115	-0.501364911	-2.1363687	0.788235184	-0.343301947	-1.653196372
284306	0.730652754	-0.452742172	-2.0078074	0.707642685	-0.498907021	-2.085183272
51665	0.668900733	-0.580135969	-2.3356637	0.76350016	-0.389299635	-1.785779506

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